Differentiation and Function of Human Th17 Cells and the Role of their Master Regulator RORC2 in T Cell Polarization

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SUMMARY

T helper (Th) 17 cells represent a recently discovered subset of T helper cells. They are characterized by the secretion of mainly pro-inflammatory cytokines like TNF-α, IL-6, IL-17A, IL-17F, IL-21, IL-22 and IL-26, and depend on the transcription factor RORC2 for their development. Th17 cells appear to be crucial for the defense of certain bacteria like *Bacteroides fragilis*, but also for immunity against parasites and yeast such as *Toxoplasma gondii* or *Candida albicans*. On the other hand, they are involved in the pathogenesis of many inflammatory and autoimmune disorders like rheumatoid arthritis, inflammatory bowel disease and psoriasis. Furthermore, Th17 seem to contribute to the inflammation in allergic diseases like allergic asthma and atopic dermatitis. While several studies investigated differentiation of Th17 cells in mice, little is known about the factors influencing development of human Th17 cells, despite their central role in a wide range of diseases.

Regulatory T (Treg) cells, in contrast, are crucial to regulate duration of immune responses as well as to establish tolerance to self- and non-self antigens. Failure in either development or function of Treg cells may lead to autoimmune and allergic disorders. Treg cells require the transcription factor FOXP3 for both their development and function. Mutations in the *foxp3* gene impair the development of Treg cells and cause a lymphoproliferative disease known as immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX). However, beside their beneficial role in suppression of inappropriate or excessive immune responses, Treg cells can also detrimentally interfere with the important immune surveillance against cancer.

Given that Th17 and Treg cells have both beneficial and adverse roles, it is crucial to balance their number and activity. Differentiation of naïve T cells towards a certain subset must therefore be tightly controlled in order to establish equilibrium between protective immune responses and tolerance to harmless self-antigens. A disturbed balance between inflammatory and regulatory T cells is often observed in patients suffering from autoimmune or allergic disorders. It is therefore of great interest to understand the mechanisms regulating this equilibrium, in order to develop drugs aiming to modulate the proportions of the different subsets.

This thesis investigates development and function of human Th17 cells and analyzes mutual cross-regulation of Th17 and Treg cells during T cell differentiation. Our data reveal that both Treg and Th17 cells depend on TGF-β for their development, while Th17 cells additionally require the inflammatory cytokines IL-1β, IL-6 and IL-23. TGF-β is necessary and sufficient to induce the transcription factor RORC2, which we find to be expressed in both subsets. Despite these similarities, *in vitro*-differentiated Th17 cells significantly differ from Treg and other T helper cells in their potency to induce IL-6 and IL-1β expression in primary bronchial epithelial cells.

In a second part, this thesis reveals that RORC2 negatively regulates FOXP3 expression in a DNA-binding-dependent manner. T cells with down-regulated levels of RORC2 develop a Treg-like phenotype even under Th17-favoring conditions, demonstrating a role for RORC2 in the polarization of Th17 versus Treg cells.

Taken together, this study reveals mechanism of Th17 and Treg differentiation and provides a basis for the elaboration of therapies aiming to restore the balance between tolerance and immunity.

ZUSAMMENFASSUNG

T Helfer (Th) 17 Zellen wurden kürzlich als eigenständige Untergruppe von T Helferzellen entdeckt. Sie sekretieren die vorwiegend entzündungsfördernden Zytokine TNF-α, IL-6, IL-17A, IL-17F, IL-21, IL-22 und IL-26. Ihre Entwicklung und viele ihrer Funktionen werden durch den Transkriptionsfaktor RORC2 gesteuert. Th17 Zellen sind unentbehrlich für die Abwehr von gewissen Bakterien wie zum Beispiel *Bacteroides fragilis*, aber auch für den Schutz gegen Parasiten und Hefepilze wie *Toxoplasma gondii* und *Candida albicans*. Auf der anderen Seite können sie viele Entzündungs- und Autoimmunkrankheiten mitverursachen, darunter rheumatoide Arthritis, Morbus Crohn und Psoriasis. Im Weiteren scheinen Th17 Zellen auch in allergischen Erkrankungen wie zum Beispiel in allergischem Asthma und atopischer Dermatitis eine Rolle zu spielen, indem sie zur Entzündung beitragen. Während mehrere Forschungsarbeiten über die Entwicklung von Maus-Th17 Zellen veröffentlicht worden sind, ist sehr wenig über die Entwicklung von menschlichen Th17 Zellen bekannt - trotz ihrer Schlüsselrolle in vielen Krankheiten.

Regulatorische T (Treg) Zellen, im Gegensatz dazu, kontrollieren die Länge einer Immunantwort und spielen eine Rolle bei der Entwicklung von Toleranz gegenüber eigenen und fremden Antigenen. Eine Störung in der Ausbildung oder Funktion dieser Zellen führt möglicherweise zu verschiedenen Autoimmun- und Entzündungskrankeiten. Die Entwicklung und Funktion von Treg Zellen wird weitgehend vom Transkriptionsfaktor FOXP3 reguliert. Mutationen im *foxp3* Gen beeinträchtigen die Entwicklung von Treg Zellen und verursachen eine X-chromosomal vererbte Krankheit, bekannt als Immundysregulation, Polyendokrinopathie und Enteropathie, (IPEX). Neben ihrer positiven Rolle in der Unterdrückung von übermässigen und unangemessenen Immunantworten können Treg Zellen dem menschlichen Körper allerdings auch schaden, indem sie die wichtige Kontrolle des Immunsystems unterdrücken, zum Beispiel bei der Entstehung von Krebs.

Aufgrund der Tatsache, dass sowohl Th17 wie auch Treg Zellen einerseits unentbehrlich sind, andererseits aber auch Krankheiten mitverursachen können, untersteht ihre Entwicklung einer strengen Kontrolle. Dadurch kann das Gleichgewicht zwischen schützender Immunantwort und Toleranz gegenüber harmlosen Antigenen aufrecht erhalten werden. In Patienten, die an

Autoimmun- oder allergischen Krankheiten leiden, ist dieses Gleichgewicht zwischen inflammatorischen und regulatorischen Zellen häufig gestört. Es ist deshalb von grossem Interesse, die Mechanismen, die dieses Gleichgewicht regulieren, zu verstehen. Dieses Verständnis hilft, Therapien zu entwickeln, die zum Ziel haben, das Verhältnis der einzelnen T Zell Untergruppen zu beeinflussen.

Die vorliegende Dissertation analysiert die Entwicklung und Funktion von menschlichen Th17 Zellen und untersucht die gegenseitige Regulation von Th17- und Treg Zellen während ihrer Differenzierung. Unsere Resultate zeigen, dass beide Zelltypen TGF-β brauchen, um sich zu entwickeln, während Th17 Zellen zusätzlich auf die inflammatorischen Zytokine IL-1β, IL-6 und IL-23 angewiesen sind. TGF-β ist notwendig und ausreichend um die Expression des Transkriptionsfaktors RORC2 zu induzieren, den wir nicht nur in Th17, sondern auch in Treg Zellen nachweisen können. Trotz diesen Gemeinsamkeiten unterscheiden sich Th17 Zellen grundlegend von Treg und anderen T Helferzellen in ihrer Fähigkeit, primäre bronchiale Epithelzellen zur Produktion von IL-1β und IL-6 anzuregen.
In einem zweiten Teil zeigt diese Arbeit, dass RORC2 als negativer Regulator des Transkriptionsfaktors FOXP3 agiert. T Zellen mit verminderter RORC2 Exprimierung entwickeln sich auch unter Th17-begünstigenden Bedingungen zu Treg-ähnlichen Zellen. Diese Beobachtung legt eine wichtige Rolle von RORC2 in der Polarisierung von T Zellen nahe.
Zusammenfassend lässt sich sagen, dass die vorliegende Dissertation Mechanismen der Th17- und der Treg-Entwicklung beschreibt und damit eine Grundlage für die Entwicklung von Therapien zur Wiederherstellung des Gleichgewichts zwischen Toleranz und Immunität bereitstellt.

ABBREVIATIONS

ActD	Actinomycin D
AIDS	Acquired immunodeficiency syndrome
AML1	Acute myeloid leukemia 1
AP-1	Activator protein 1
APC	Antigen-presenting cell
BALT	Bronchial-associated lymphoid tissue
BFA	Brefeldin A
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CREB	Cyclic AMP response element-binding protein
DAPI	4',6-Diamidino-2-phenylindol
DBD	DNA-binding domain
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EF-1α	Elongation factor 1α
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial cell-derived neutrophil-activating protein 78
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box protein 3
GATA-3	GATA-binding protein 3
G-CSF	Granulocyte colony stimulatory factor
GM-CSF	Granulocyte monocyte colony stimulatory factor
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN-γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IP-10	IFN-γ-inducible protein 10
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF	Interferon regulatory transcription factor
iTreg	Induced T regulatory cell
LBD	Ligand-binding domain
LTα	Lymphotoxin-α
MACS	Magnetic-activated cell sorting
MPC-1	Monocyte chemoattractant protein
MHC	Major histocompatibility complex

mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NALT	Nasal-associated lymphoid tissues
NFAT	Nuclear factor of activated T cells
NHBE	Normal human bronchial epithelial
NK	Natural killer
NKT	Natural killer T
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-cyanine 5
PMA	Phorbol 12-myristate 13-acetate
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
ROR	RAR-related orphan receptor
RORE	ROR-response element
RT-PCR	Real-time polymerase chain reaction
RUNX1	Runt-related transcription factor 1
SCID	Severe combined immunodeficiency
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
T-bet	Th1-specific T box transcription factor
Tc	T cytotoxic
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
TNF-α	Tumor necrosis factor α
Treg	Regulatory T
TSS	Transcription start site

1 INTRODUCTION

1.1 Adaptive immunity

Mechanisms to defend the host against harmful microbes exist in all multicellular organisms. While the defense of invertebrates constitute a system called innate immunity, vertebrates have evolved a second, more specialized system - the adaptive immunity - , which tightly interacts with innate immunity.

Both innate and adaptive immunity depend on the ability to distinguish harmless self (parts of the own body) and non-self (foreign substances). In a healthy organism, tolerance of self-antigens and immunity against pathogens are well balanced. Under certain pathological conditions, however, this delicate equilibrium is disturbed. The lack of an efficient immune response can result in fatal infections. Such an immunodeficiency is observed in genetic diseases like severe combined immunodeficiency (SCID), or in patients infected with the human immunodeficiency virus (HIV) causing the acquired immunodeficiency syndrome (AIDS). In contrast, inappropriate responses against self can lead to autoimmune disorders like inflammatory bowel disease or rheumatoid arthritis. Similar malfunctions are observed in allergic diseases, where an excessive immune reaction is raised against a harmless antigen, which can have severe consequences for the allergic individual.

While the innate immunity sets up an unspecific response to a broad range of invaders, the adaptive immunity responds to the challenge with a high degree of specificity. Furthermore, the adaptive immunity has the ability to build a memory, enabling it to respond more vigorously when exposed to the same microbe a second time. Specificity and memory therefore represent characteristic features of the adaptive immunity and are mediated by a group of immune cells called lymphocytes.

1.1.1 Players of the adaptive immunity

Lymphocytes can be divided into two major populations: B lymphocytes and T lymphocytes. B lymphocytes mature in the bone marrow and are responsible for the production of

antibodies. Antibodies facilitate elimination of microorganisms by phagocytotic cells and activate the complement system, leading to lysis of the pathogen. Furthermore, they can also neutralize toxins or viral particles by coating and preventing them to bind to host cells.
T lymphocytes also arise in the bone marrow, but mature in the thymus. There are two distinct T lymphocyte populations, the T cytotoxic (Tc) cells and the T helper (Th) cells, which can be distinguished by the expression of their characteristic surface molecules CD8 and CD4, respectively. Tc cells can directly kill infected cells through release of molecules like granzymes or perforin, while Th cells are responsible for the activation of other immune cells by secreting cytokines. Differences in the cytokine pattern secreted by Th cells result in different immune responses and therefore decide about the success of the reaction.
Th cells that leave the thymus are called naïve, as they have not yet been in contact with foreign antigens. Before these cells are able to stimulate other cells by cytokine secretion, they need to be activated and differentiated themselves.

1.1.2 Activation and differentiation of Th cells

Activation of Th cells is initiated by contact with antigens presented by antigen-presenting cells (APC). APC include macrophages, B lymphocytes and dendritic cells (DC). DC are are particularly important for the activation of naïve T cells, and they are specialized to capture and process antigenic material in the periphery and transport it to the secondary lymphoid organs. Pathogens taken up by a DC are processed into short peptides. Together with a major histocompatibility complex (MHC) molecule, these antigenic peptides then form a complex, which is displayed on the DC's cell surface. The DC undergoes a maturation step and migrates to a lymph node, where it presents the peptide/MHC complex to naïve T cells that continuously recirculate through these organs. If the T cell receptor (TCR) of a naïve T cell specifically binds to the peptide/MHC complex, signaling cascades are induced, leading to activation, differentiation and proliferation of the T cell. In addition to the signals delivered by TCR stimulation, a second signal via costimulatory molecules displayed by the DC is required for a successful activation. Examples for such molecules are CD80 and CD86, which interact with CD28 on T cells, or ICOS-L, which binds to ICOS. Importantly, also cytokines secreted by DC and surrounding cells contribute to the development of the T cell and

modulate its functional properties. The composition of the cytokines secreted by the DC depends on the encountered pathogen. Therefore, DC activate naïve T cells by presenting the antigen together with a costimulatory signal and factors that tailor the properties of the T cells according to the pathogens to be defeated. Upon stimulation, the activated T cell rapidly divides. This process called clonal expansion gives rise to a substantial population of T cells all directed against the same antigen and with qualities appropriate to repel the corresponding pathogen. Together, these mechanisms mount an effective Th cell-mediated immune response.

1.1.3 Th cell subsets

In 1986 Mosmann and Coffman discovered that Th cells are not a uniform population, but can be subdivided into two groups, one making IFN-γ and the other producing IL-4 (1). The IFN-γ-secreting subset has been termed Th1 cells, while IL-4-producing cells were named Th2 cells. This Th1/Th2 paradigm has recently been complemented with a third effector T cell subset, the Th17 cells, named after their hallmark cytokine IL-17 (2, 3). In addition to these effector cell lineages, a population characterized by regulatory or suppressive activity, called Treg cells, exists (4).

Although these subsets are marked by distinct features, some of their properties are overlapping, and subsets with mixed phenotypes seem to occur. Furthermore, it is likely that much more than three effector cell subsets exist. Accordingly, it has been proposed that a population of IL-9-producing T cells ~~constitute~~ a separate subset, called Th9 cells (5, 6). Furthermore, three very recent reports suggest the existence of Th22 cells, a subset secreting IL-22, which has previously been associated with Th17 cells (7-9).

The differentiation towards a certain subset depends on the cytokines present in the microenvironment during stimulation of a naïve T cell with an antigen presented by an APC (Fig 1). Once differentiated, each Th population secretes a confined set of cytokines and has distinct functions in the immune system. Furthermore, the subsets express specific transcription factors that regulate their development.

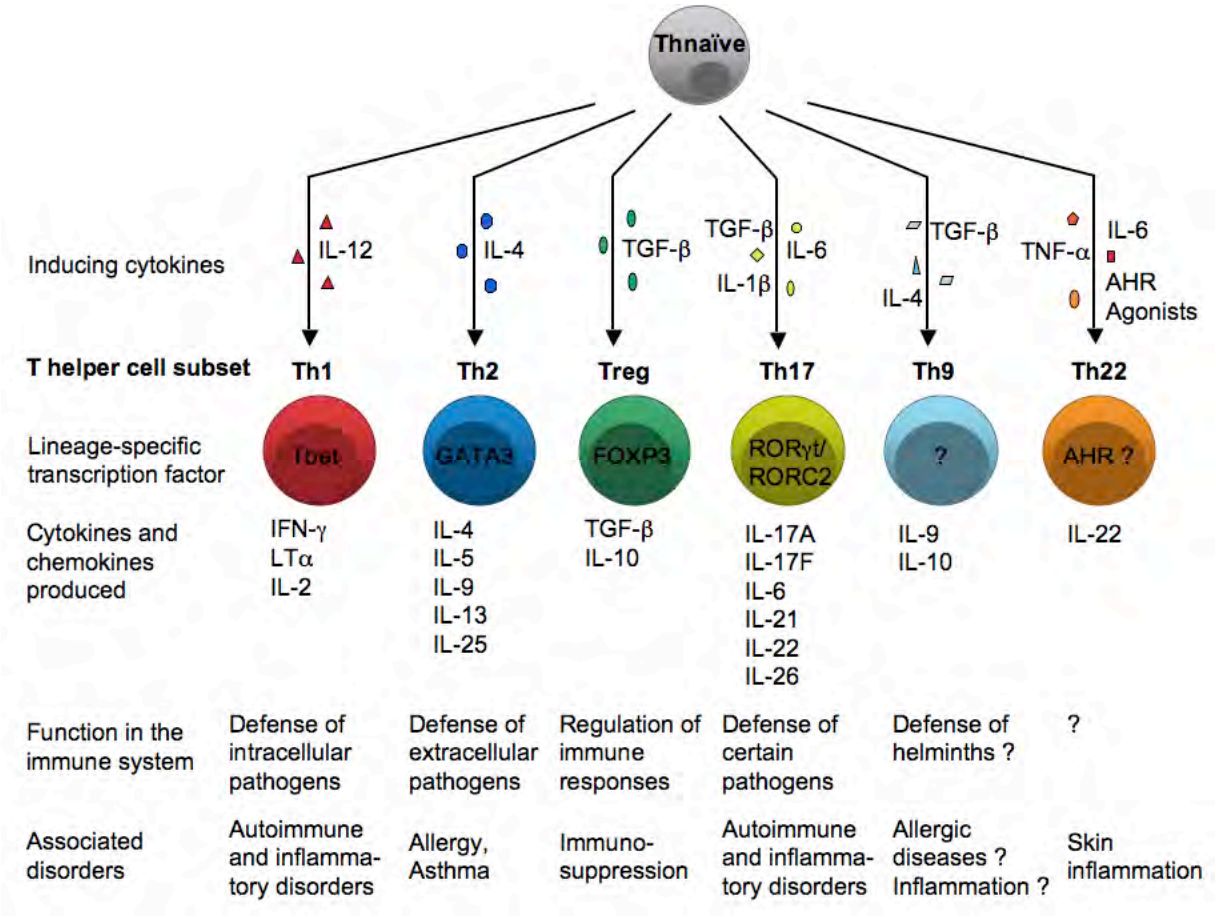

Fig 1. Differentiation of T cell subsets. Naïve T helper cells differentiate towards distinct subsets, depending on the cytokines present during antigenic stimulation. The differentiation pathways are controlled by lineage specific transcription factors. Each subset secretes a characteristic set of cytokines and exerts specific functions.

1.1.3.1 Cross-regulation of Th cell subsets during polarization

Cytokines secreted by a certain T cell subset not only determine the effector function of the subset, but also act in an autocrine manner to give a positive feedback and promote development and expansion of the subset. Some cytokines also enhance the expression of their receptors and thereby increase the responsiveness of the cell. In addition, cytokines secreted by one subset can inhibit differentiation of other subsets. The initiated T cell population thus gets increasingly defined and dominant with every cell division. These

mechanisms of T cell polarization ensure expansion of the desired population and are crucial for mounting an effective immune response.

Mutual inhibition of T cell subpopulations is also mediated by the lineages specific transcription factors (Fig 2). The Th1 cell transcription factor T-bet inhibits Th2 development by binding to their master regulator GATA-3, preventing it from activation of Th2-specific genes (10, 11). Likewise, GATA-3 can down-regulate STAT4, a transcription factor important for Th1 development (12). GATA-3 also negatively regulates FOXP3 expression and thereby inhibits Treg development (13). FOXP3 in turn suppresses Th17 development by inactivating their transcription factor RORC2 through direct interaction (14, 15). Transcription factor-mediated cross-inhibition of T cell subsets during differentiation is therefore an important mechanism of T cell polarization.

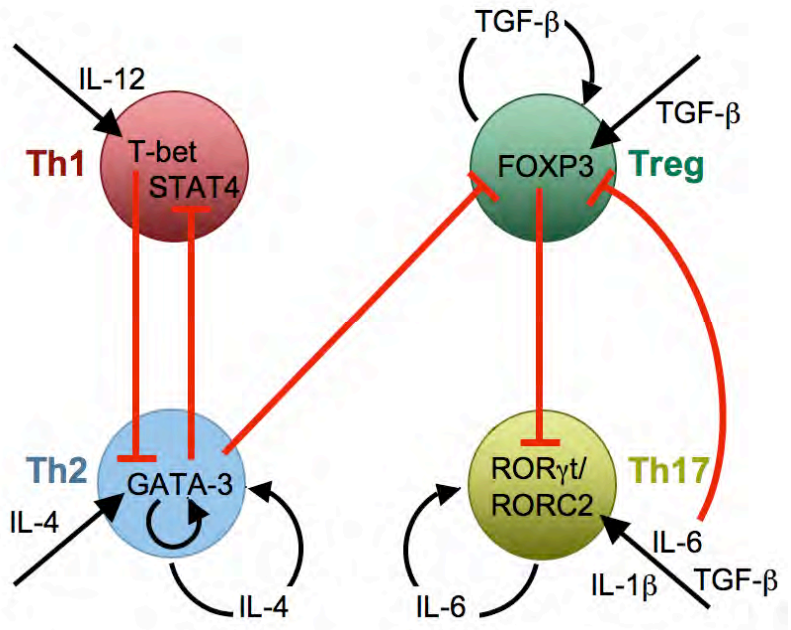

Fig 2. Th cell cross-regulation. Cytokines act in an autocrine manner to give a positive feedback on the development of their subset, while inhibiting differentiation of other subsets. Similarly, the lineage-specific transcription factors mutually inhibit their expression or function. These mechanisms ensure selective expansion of the initiated population.

1.1.3.2 Th1 cells

Th1 cells require IL-12 signaling for their development. IL-12 acts via signal transducers and activators of transcription (STAT) 1, which up-regulates the Th1-specific transcription factor T-bet (16, 17). T-bet is important for the induction of IFN-γ production and enhances expression of IL-12Rβ2. Up-regulation of IL-12Rβ2 in turn renders the developing Th1 cell more responsive to IL-12, thereby promoting full Th1 differentiation.

Th1 cells are important for the defense of intracellular pathogens such as mycobacteria and viruses. They exert these functions mainly by activating and recruiting other immune cells ~~trough~~ secretion of cytokines and chemokines like IFN-γ, lymphotoxin-α (LTα) and IL-2. IFN-γ produced by Th1 cells is important to enhance microbicidal activity of macrophages (18). Excessive function of Th1 cells, however, is a cause of many autoimmune and inflammatory disorders, as Th1 cytokines can induce severe inflammation and tissue damage.

1.1.3.3 Th2 cells

Th2 cells depend on IL-4 for their development (19). Signaling through STAT6, IL-4 induces low levels of the Th2 cell master regulator GATA-3 (20). GATA-3 auto-activates its own expression and induces Th2-specific cytokines like IL-4, IL-5 and IL-13 (21, 22). STAT5 is another important transcription factor in Th2 development, as IL-2-activated STAT5 induces the production of IL-4 (23).

Secreting the cytokines IL-4, IL-5, IL-9, IL-13 and IL-25 (also known as IL-17E), Th2 cells are responsible for the defense of extracellular pathogens like helminths. IL-4 is important not only for a positive feedback on Th2 development, but also for IgE class switching in B cells (24). IL-5 and IL-13 play a crucial role in the recruitment of eosinophils upon helminth infection and in the expulsion of these parasites (25, 26). However, when disregulated, Th2 functions can lead to allergic disorders like allergy and asthma.

1.2 Treg cells

1.2.1 Function of Treg cells

T cells with suppressive capacity have been described already in 1972 (27), and it now becomes apparent that during every adaptive immune response not only effector, but also regulatory T cells are activated and recruited. Induction of Treg cells appears to be important to control the strength and duration of the response as well as to establish tolerance to self- and non-self antigens. Failure in either development or function of Treg cells is often observed in autoimmune and inflammatory disorders, as demonstrated in many mouse models. For instance, Treg-depleted T cell suspensions transferred into nude mice evoke autoimmune disease, which can be prevented by cotransfer of a small number of Treg cells (28, 29). In another experiment, depletion of Treg induced inflammatory bowel disease as an inappropriate reaction to commensal bacteria, demonstrating a role for Treg cells in prevention of inflammatory diseases in response to foreign antigens (30). Treg cells isolated from normal mice have been shown to suppress allergy and to confer tolerance to organ grafts (31). In ~~human,~~ Treg cells play an important role in induction of specific tolerance towards allergens (32, 33).

In contrast to the beneficial role of Tregs in suppression of inappropriate or excessive immune responses, they can detrimentally interfere with the important immune surveillance against cancer by inducing tumor-specific local immune tolerance (34).

Treg cells have been shown to suppress the development, proliferation and/or activity of a wide range of cells, among them Th and Tc cells, natural killer (NK) cells, natural killer T (NKT) cells, DC, B cells, macrophages and osteoclasts (35-38). Treg cells mediate suppression of these effectors by a not yet defined cell-cell contact dependent mechanism, which either involves the immunosuppressive cytokines TGF-β and IL-10 (39-42), or is cytokine-independent (43-45). As an alternative, Treg cells may compete with effector T cells for cytokines and thereby induce deprivation-mediated apoptosis in these cells (46). Treg cells have also been suggested to kill other cells via granzyme B or perforin (38). Other studies report that Treg cells deliver inhibitory signals, either directly to T cells, or indirectly by downregulating costimulatory molecules on APC (38).

The mechanism of suppression probably depends on the location of the inflammation, but also on the type of Treg cells.

1.2.2 Subtypes of Treg cells

Several subtypes of Treg cells have been reported. Tr1 cells are characterized by the production of IL-10 and TGF-β. *In vitro*, they can be differentiated by antigen-stimulation in the presence of IL-10. Suppressive capacity of Tr1 cells has been demonstrated in a mouse model of colitis (47), and in ~~human~~ they play an important role in allergies and transplantation (48-51). Antigen-specific regulatory cells, called Th3 cells, were originally discovered in a mouse model of oral tolerance (52). They seem to play a suppressive role not only in many experimental autoimmune models, but also in human autoinflammatory diseases (53, 54). Th3 regulatory cells are triggered in an antigen-specific fashion but suppress in an antigen-non-specific fashion (55). Tr1 and Th3 cells belong to the group of induced Treg cells, and the role and significance of these Tregs *in vivo* is not well understood.

More research has been done on natural Treg cells. This subset of regulatory cells is characterized by the expression of the surface molecules CD4 and CD25, and by high levels of forkhead box protein 3 (FOXP3), a transcription factor that plays a key role in Treg cell-mediated suppression. Natural Treg cells develop in the thymus and are, unlike most other thymus-generated naïve T cells, already functionally mature before leaving the thymus. Evidence suggests that interaction of TCR with self-peptide-MHC on thymic stromal cells is important for the generation of natural Tregs. T cells in the periphery can also acquire FOXP3 expression and suppressive capacity. This has been demonstrated by stimulating naïve T cells with antigen in the presence of TGF-β *in vitro* (4, 56, 57). Beside TGF-β, retinoic acid has been shown to facilitate development of Treg cells in peripheral tissues like in the gut (58-61). However, Treg cells induced in the periphery are presumably functionally less stable than natural Treg, as suggested by a lower and more transient expression of FOXP3 (62, 63).

1.2.3 The transcription factor FOXP3

FOXP3 belongs to the forkhead/winged helix transcription factor family and plays a central role in development and function of Treg cells. Initially, FOXP3 ~~has been~~ identified as a gene defective in scurfy mice, an X-linked mutant that is lethal in male mice (64). In scurfy mice, a frameshift mutation results in a product lacking the forkhead domain, leading to a non-functional FOXP3 protein and hence to an abnormal immune homeostasis characterized by hyperactivation of T cells and cytokine overproduction. The importance of FOXP3 is further illustrated by the human genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), caused by mutations in the *foxp3* gene (65, 66). While female carriers of a mutant X-allele stay healthy, male IPEX-patients suffer from a lymphoproliferative disease with immunological and clinical symptoms resembling the symptoms of scurfy mice.

1.2.3.1 Suppressive function of FOXP3

The observation that disruption of FOXP3 impairs immune regulation initiated studies investigating the role of FOXP3 in Treg function. Retroviral gene transfer of FOXP3 has been found to be sufficient for the conversion of naïve T cells into a regulatory T cell phenotype similar to that of natural regulatory T cells (67). Furthermore, overexpression of FOXP3 confers suppressor function on peripheral $CD4^+CD25^-$ T cells (68, 69). These reports strongly suggest a key role for FOXP3 in the development and function of regulatory T cells.
Despite this strong evidence, little is known about the function of FOXP3 on molecular level. However, FOXP3 has been reported to interact with transcription factors like nuclear factor of activated T cells (NFAT) or acute myeloid leukemia 1/runt-related transcription factor 1 (AML1/RUNX1) (70, 71). As these transcription factors facilitate activation of effector T cells, FOXP3 might exert suppressive function by binding and thereby inhibiting the activity of these transcription factors. Similarly, two recent studies describe binding of FOXP3 to retinoic acid receptor-related orphan receptor γt (RORγt), the master regulator of Th17 cells

(14, 15). Binding of FOXP3 to RORγt inhibits its transcriptional activation of the Th17 developmental program, including production of IL-17.

Genome-wide searches for FOXP3 target genes revealed that FOXP3 regulates approximately 700 genes, either by binding directly to their promoters, or indirectly (72, 73). Interaction of FOXP3 with these genes can induce both transcriptional suppression or activation. Given this important role as activator and repressor of a wide range of genes, several studies investigated the mechanism regulating FOXP3 expression.

1.2.3.2 Transcriptional regulation of FOXP3

Signals downstream TCR stimulation are necessary for the induction of FOXP3. Studies from our lab revealed an activatory region in the proximal promoter of the *foxp3* gene (74). This region contains binding sites for NFAT and activator protein 1 (AP-1), which contribute to the TCR-induced FOXP3 activation (Fig. 3). Additionally, cAMP response element-binding protein/activating transcription factor (CREB/ATF) has been shown to bind to an intronic enhancer element of the *foxp3* gene, thereby promoting its transcription (75).

However, TCR-stimulation appears not to be sufficient for high FOXP3 expression and IL-2 seems to play a major role in providing a second signal for the induction of FOXP3 (76, 77). IL-2-mediated activation of the *foxp3* gene is likely to be mediated by the transcription factor STAT5, as binding sites for STAT5 were found on the FOXP3 promoter (78). The importance of STAT5 is further underlined by a report identifying a patient carrying a homozygous mutation in the *stat5b* gene, which leads to impaired FOXP3 expression and Treg function (79). In addition to IL-2 and other cytokines of the common γ-chain family, TGF-β plays a central role in the induction of FOXP3 expression. The mechanism possibly involves signaling via the transcription factor Smad3, which has been shown to activate an enhancer region of the *foxp3* gene (80). In addition, TGF-β decreases methylation of a CpG island in the first intron of the *foxp3* gene, which leads to enhanced transcription (75).

Interestingly, cytokines involved in Th1 cell development also positively regulate the *foxp3* gene. IFN-γ, for example, takes part in the induction of FOXP3 and the conversion of conventional T cells into Treg cells in EAE (81). Our group recently demonstrated that IL-27 enhances TGF-β-induced FOXP3 expression in a STAT1-dependent manner (82). IL-27-

induced STAT1 binds to the proximal promoter of FOXP3 and induces permissive histone acetylation leading to an increased transcription of the *foxp3* gene. Beside STAT1 and STAT5, also STAT3 seems to positively or negatively influence FOXP3 expression (82-85).

FOXP3 expression and Treg cell development ~~is~~ enhanced by the lipidic mediator retinoic acid (60, 86-88). Retinoic acid is a ligand for the retinoic acid receptor (RAR) that binds to the FOXP3 promoter and positively regulates its expression (89).

In contrast, a suppressive role in FOXP3 regulation has been found for the Th2-specific transcription factor GATA-3 (13). This study performed in our laboratory revealed that IL-4-induced GATA-3 binds to a palindromic GATA-site in the FOXP3 promoter, which leads to transcriptional repression. IL-4 activates also STAT6, which then binds to the FOXP3 promoter and inhibits gene expression (89). Furthermore, FOXP3 expression is suppressed by the cytokines IL-1β, IL-6 and IL-21 (90-92). While IL-6 mediates its inhibitory function through methylation of a CpG island in the promoter (93), the ~~mechanism~~ by ~~with~~ IL-1β and IL-21 suppress FOXP3 expression remain to be determined.

IL-1β, IL-6 and IL-21 are required for the development of Th17 cells. As FOXP3 inhibits Th17 cell development (14, 15), suppression of FOXP3 by these cytokines may be an important mechanism of Th17 development.

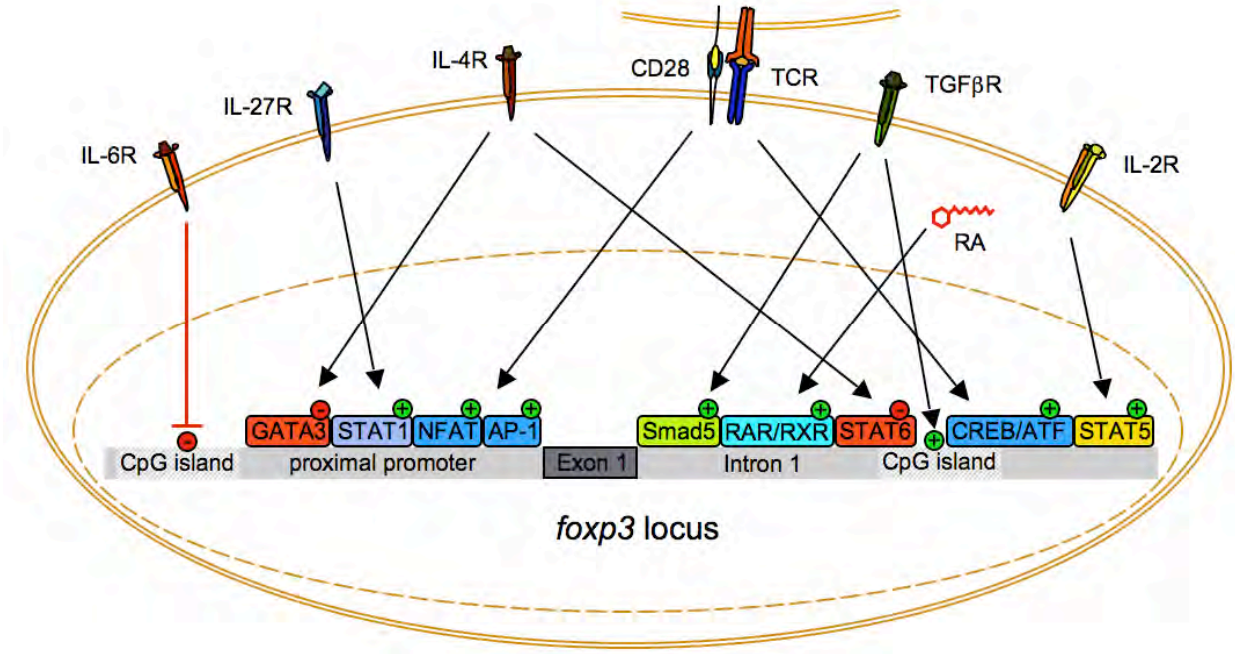

Fig 3. Transcriptional regulation of the *foxp3* gene. TCR stimulation as well as cytokine signaling leads to activation of transcription factors that positively or negatively regulate transcription of FOXP3. RA: retinoic acid.

1.3 Th17 cells

Analysis of various T cell clones revealed that IL-17A-producing T cells cannot be classified into Th1 or Th2 subsets, but rather represent a distinct cell subset (2, 3, 94, 95). Subsequently, this subset was described to secrete also IL-17F, IL-6, IL-21, IL-22, IL-26 and TNF-α, and has been termed Th17 (92, 97-99).

1.3.1 Function of Th17 cells

Receptors for Th17-cytokines are expressed on many non-immune cells like fibroblasts, epithelial cells, endothelial cells, keratinocytes or marrow stromal cells, and can be found in a wide range of tissues such as kidney, liver, intestine, colon, lung, pancreas and skin (100-102). Upon stimulation with Th17-cytokines, structural cells respond with the production of pro-inflammatory cytokines, chemokines or metalloproteases, which leads to inflammation and tissue destruction (Fig 4). It becomes apparent that Th17 cells contribute to the inflammation of many inflammatory and autoimmune disorders by secreting cytokines that induce pro-inflammatory mediators in cells of the affected tissues.

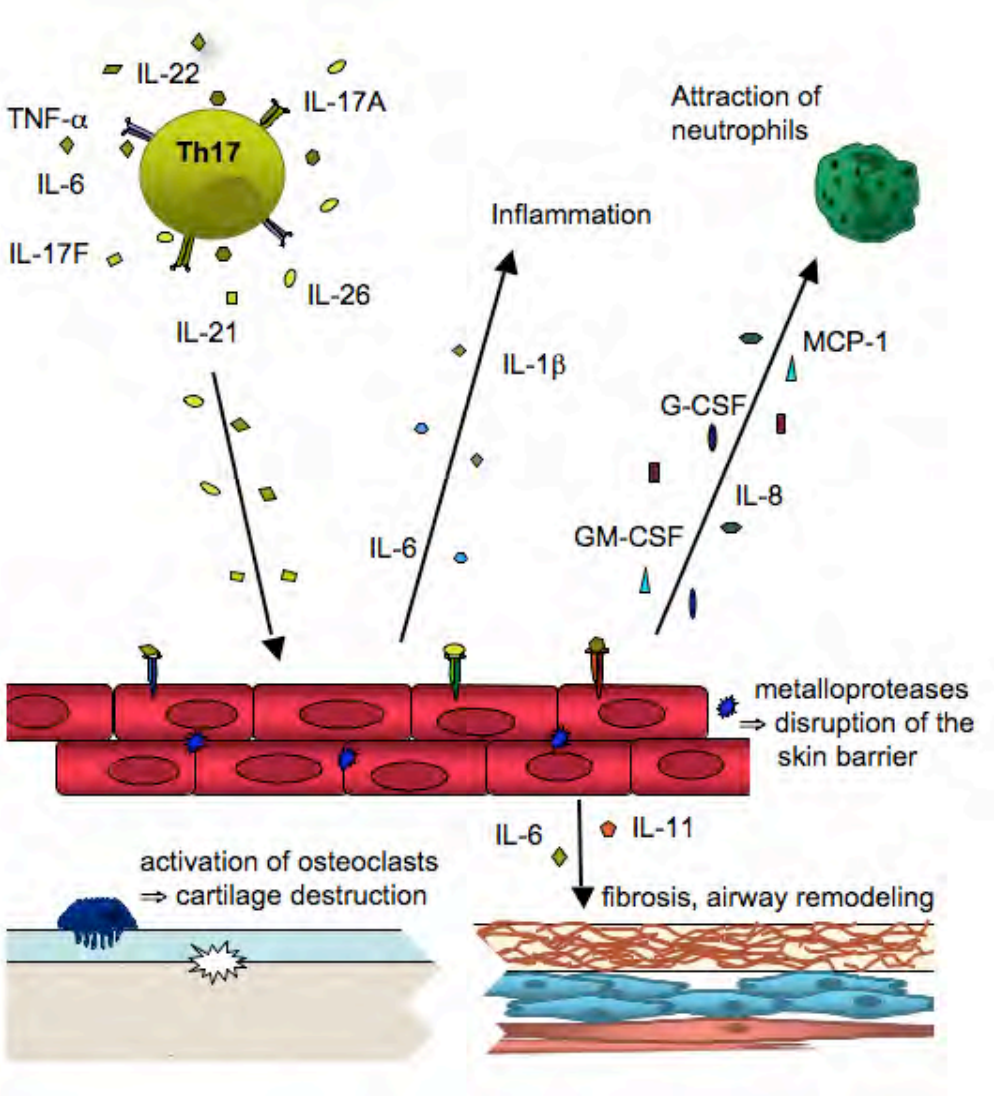

Fig 4. Pathogenic mechanisms of Th17 cells. Cytokines produced by Th17 cells act on a wide range of tissue cells and stimulate these cells to produce pro-inflammatory and pro-fibrotic cytokines and chemokines. These mediators induce attraction and activation of neutrophils and tissue destruction.

1.3.1.1 Effects of Th17 cell cytokines on tissue cells

IL-17A was initially described as potent inducer of IL-6 and IL-8 (CXCL8) in fibroblasts (102, 103). Subsequently, IL-17A has been reported to induce other CXC-chemokines like CXCL1 (Gro-α), CXCL6, CXCL10 and CINC in different cells (104-107). Furthermore, IL-17A has been shown to induce the colony-stimulating factors GM-CSF and G-CSF, and monocyte chemotactic protein (MCP)-1 in intestinal and bronchial epithelial cells (107-109).

The induction of chemoattractants is in accordance with a study reporting a high potential for IL-17A in the recruitment and activation of neutrophils (110). Tissue fibroblasts and bronchial epithelial cells release IL-6 and IL-11 upon stimulation with IL-17A (111). IL-17A seems to act also on immune cells as certain monocytes respond by secreting TNF-α and IL-1β as well as metalloproteases (112, 113).

IL-17F was shown to induce IL-6, IL-8, CXCL1 (Gro-α) and epithelial cell-derived neutrophil-activating protein (ENA)-78 (114, 115). Endothelial cells stimulated with IL-17F respond with increased expression of IL-2, TGF-β and monocyte chemoattractant protein (MPC)-1 (116). Other studies report induction of MIP-1beta/CCL4, IL-1β, G-CSF, GM-CSF and IFN-γ-inducible protein 10 (IP-10) (117-120). IL-22 up-regulates production of IL-10, acute phase reactants and anti-microbial peptides in keratinocytes and in several cell lines (101, 121, 122), while IL-26 has been shown to induce pro-inflammatory cytokines like TNF-α, IL-6 and IL-8 in intestinal epithelial cell (IEC) lines (123).

1.3.1.2 Th17 cells in inflammatory diseases

Elevated levels of IL-17A mRNA have been found in blood and cerebrospinal fluid of patients with multiple sclerosis, ~~but the role of IL-17A in this disease is not completely clear.~~ Studies using the corresponding mouse model experimental autoimmune encephalomyelinitis (EAE) showed that Th17 cells are responsible for the inflammation in this disease (96), and that IL-17A and IL-22 promote blood-brain barrier disruption, kill neurons and promote central nervous system inflammation through lymphocyte recruitment (124). IL-17A and IL-17F play also a role in rheumatoid arthritis (125) as well as in the corresponding mouse model collagen-induced arthritis (126, 127). The pathogenic mechanism seems to involve IL-17A-mediated induction of metalloproteases and activation of osteoclasts, which leads to destruction of bones and cartilage (128).

Increased levels of IL-17A are also found in biopsies from inflamed colonic tissue of inflammatory bowel disease patients (129). IL-17A stimulates production of matrix metalloproteases and pro-inflammatory cytokines in colonic subepithelial myofibroblasts (130) and inhibits the proliferation of intestinal epithelial cells (131), as does IL-26 (123).

This suggests that Th17 cells not only contribute to inflammation and tissue damage in IBD, but also interfere with the repair mechanism important for maintenance of tissue integrity.
Th17 cells seem to be involved in psoriasis, as increased levels of IL-17A have been found in psoriatic patients (132). Similarly, IL-22 mediates dermal inflammation and acanthosis and is required for skin inflammation in a mouse model of psoriasis (133, 134). IL-17A and IL-22 induce anti-microbial peptides and matrix metalloproteases that are commonly found in psoriatic skin (97, 122). Th17 cells might therefore contribute to skin inflammation via induction of these mediators. Hints for a role of Th17 cells in allergic skin diseases come from studies describing Nickel-specific Th17 cells (135) and expression of IL-17A in acute lesions of atopic dermatitis patients (136). The mechanism by which Th17 cells contribute to these diseases needs to be determined.
Increased levels of IL-17A have been found in patients with allergic asthma (111, 137), which may explain the high numbers of neutrophilic granulocytes in allergic airways (105, 138). IL-17A has also been described to induce expression of the mucin genes MUC5AC and MUC5B, contributing to mucus hypersecretion (139). Besides, IL-17A might have a role in airway remodeling, a feature commonly observed in severe asthma, as IL-17A was shown to induce the pro-fibrotic cytokines IL-6 and IL-11 (140). Together, these reports suggest an involvement of Th17 cells in chronic inflammation in asthmatic airways, leading to irreversible changes.
IL-26 seems to be present in inflamed tissue in general (141), but its role in disease is not clear yet and functional investigations are complicated by the fact that IL-26 does not exist in mice.
Taken together, Th17 cells contribute to inflammation and tissue destruction in many diseases. However, Th17 cell-mediated inflammatory responses represent also an important defense mechanism against various pathogens.

1.3.1.3 Th17 cells in host defense

High induction of IL-17A has been reported in T cells after priming with lysates of *Borrelia burgdorferi* (95). Similarly, IL-17A has been shown to be necessary for protection from *Klebsiella pneumonia* infections (110), and to be important in the defense of other bacteria

like *Bacteroides fragilis* (142), and *Porphyromonas gingivalis* (143). In addition, IL-17A plays a role in the expulsion of parasites and yeasts like *Toxoplasma gondii* (144) or *Candida albicans* (145).

IL-22 seems to be important for the defense of *Citrobacter rodentium* (146). Furthermore, IL-22 levels increase upon infection with *Mycobacterium tuberculosis* and *Klebsiella pneumoniae*, suggesting a role for IL-22 in the immune response against these bacteria (147, 148). The mechanisms of IL-17A- and IL-22-mediated host defense differ: while IL-17A confers immunity against pathogens by attracting neutrophils (110), IL-22 enhances the expression of β-Defensins, psoriasin, calgranulin A and calgranulin B in keratinocytes, thereby increasing their antimicrobial defense (101, 122).

1.3.3 Development of Th17 cells

1.3.3.1 Cytokines involved in differentiation of *murine* Th17 cells

Studies reporting that IL-17-producing cells are markedly diminished in IL-23-deficient mice, and that these mice are resistant to EAE (96, 149) suggested a key role for IL-23 in Th17 cell development. However, despite the important role of IL-23 in IL-17 production, IL-23 failed to induce IL-17 in naïve T cells, which is not surprising, as naïve T cells do not express a receptor for IL-23. Subsequently, three independent reports described that a combination of IL-6 and TGF-β can induce IL-17 production from naïve T cells (150-152). The involvement of TGF-β in the development of the inflammatory subsets of Th17 cells was a surprise, as TGF-β has been associated mainly with anti-inflammatory effects such as the development of regulatory T cells. Thus, IL-6 seems to be the factor that favors Th17 cells over Treg cells in the presence of TGF-β. Another important role of IL-6 in the Th17 development is to induce expression of the receptor for IL-23, a characteristic feature of Th17 cells (153, 154). The role of IL-23 in Th17 cells development is still not fully clear. Although IL-23 cannot induce *de novo* differentiation of Th17 cells, there is now a consent that IL-23 is important for the expansion of differentiated Th17 cells and for the stabilization of their phenotype, characterized by sustained IL-17 production (96, 152, 154-156). Furthermore, IL-23 appears to be involved in the effector function of Th17 cells, as IL-23-deficient mice are unable to elicit a protective Th17 cells response upon infection with *Citrobacter rodentium* (151).

T cells generally require cytokines of the common gamma chain family such as IL-2 for their survival. Surprisingly, Th17 cells are not dependent on IL-2, but might rather be inhibited by IL-2 (157). IL-21, another common gamma chain family member has been suggested to act as a survival factor for Th17 cells and may even substitute for IL-6 (92, 154, 158). Induced by IL-6, IL-21 is produced by Th17 cells themselves and can act in an autocrine manner. IL-1β, a pro-inflammatory cytokine like IL-6 appears to contribute to the development of Th17 cells, although it cannot substitute for IL-6 (152, 159).

Taken together, IL-6 and TGF-β are the key mediators to induce differentiation of naïve T cells towards Th17 cells in mice, a process that is enhanced by IL-1β. In addition, IL-21 and IL-23 seem to be important for the expansion of the population and for its functions such as IL-17 production.

1.3.3.2 Cytokines involved in differentiation of *human* Th17 cells

While development of mice Th17 cells has been intensively studied, the differentiation of human Th17 cells is still controversial. One report suggests a combination of IL-1β and IL-6 to be optimal for Th17 induction with IL-23 having poor effects (160). In contrast, two other groups describe an efficient induction of human Th17 cells by IL-23 (99, 161). Apart from these inconsistencies, the three groups suggested that, in contrast to mouse, TGF-β is not necessary for human Th17 cells development. Three recent reports, however, support a central role for TGF-β in human Th17 ~~cells~~ development (162-164). The discrepancies might be due to differences in culture conditions, such as usage of serum. Alternatively, different procedures of naïve T cell isolation might result in various amounts of contaminating memory cells, which may influence the results.

Similar to the observations in mice, IL-21 has been described to be involved in the development of human Th17 cells and is proposed to substitute for IL-6 under certain conditions (164).

Therefore, human Th17 cells appear to require similar cytokines for their induction, although differences might exist. However, further investigations need to be conducted to elucidate the factors regulating human Th17 cell development.

1.3.3.3 Transcriptional control of Th17 cells

One mechanism by which cytokines exert their functions is through activation of the STAT-signaling pathway. In T cell differentiation, particular STAT molecules induce the lineage-specific transcription factors T-bet, GATA3 or FOXP3. STAT3 has been reported to be a crucial component in the differentiation pathway of Th17 cells, as mice bearing increased STAT3 activity were found to have an increased IL-17 production (165). Activated by IL-23, STAT3 binds to the IL-17 promoter, thereby inducing its transcription. STAT3 signaling can also be induced by IL-6. Consistently, TGF-β- and IL-6-mediated Th17 cell differentiation is defective in STAT3-deficient mice (155). Moreover, STAT3 appears to be involved in the IL-21-mediated Th17 development (92, 154). In addition to STAT3, interferon-regulatory factor 4 (IRF4) takes part in Th17 differentiation, as IRF4-deficient mice completely lack Th17 cells and are protected from the Th17-mediated disease EAE (166).
While STAT3 and IRF4 are not exclusively expressed in Th17 cells, RORC2 (also named RORγt) has been identified as lineage specific transcription factor of Th17 cells (153, 162, 163). Overexpression of RORC2 promotes Th17 cell differentiation, while RORC2-deficient mice have an impaired Th17 response. Moreover, EAE is attenuated in these animals due to reduced numbers of Th17 cells. These findings strongly support a role for RORC2 as master regulator of Th17 cells development. However, RORC2-deficient mice still have low numbers of Th17 cells and can develop EAE. Recently, RORA (RORα) has been shown to promote Th17 cell development when overexpressed (167). Therefore, RORA might substitute for RORC2 in RORC2-deficient mice, which can explain the presence of Th17 cells in these mice.

1.3.4 The ROR-family of transcription factors

The family of retinoic acid receptor (RAR)-related orphan receptors (ROR) consists of three members: RORA (NR1F1, RORα, RZRα), RORB (NF1F2, RORβ, RZRβ) and RORC (NF1F3, RORγ, RZRγ). Each ROR gene generates several isoforms that differ only in their N-termini (168-170). Four human RORA isoforms have been identified - RORA1-4 - while only isoform 1 and isoform 4 exist in mice. Two RORB isoforms have been reported in mice

and one isoform in human. The *rorc* gene generates two isoforms, RORC1 and RORC2 (also referred to as RORγ and RORγt) in both, human and mice. Most ROR isoforms are under the control of different promoters and exhibit a tissue specific expression pattern.

Proteins of the ROR family are between 459 and 556 amino acids in length and display a typical nuclear receptor domain structure consisting of four domains: an N-terminal domain, a highly conserved DNA-binding domain (DBD) consisting of two zinc finger motifs, a hinge domain and a C-terminal ligand-binding domain (LBD). The DBD binds to DNA containing a so-called ROR-response element (RORE), which consists of the core motif GGTCA preceded by an A/T-rich sequence (171, 172). In contrast to other nuclear receptors, ROR family proteins bind DNA as monomers (169, 171, 173). The interaction is mediated by the loop between the last two cysteines in the first zinc finger, and a 30 amino acid long region downstream the two zinc fingers (169, 174). Due to the high homology of the DBD between the ROR family members and their different isoforms, they recognize closely related RORE. However, the affinities for different RORE vary between members and isoforms. This is due to the differences in their N-terminal domains, which are involved in DNA-recognition and thereby confer DNA-binding specificity (169). The LBD of ROR transcription factors is characteristic for nuclear receptors, but contains - in addition to the typical 12 α-helices - two additional helices (175, 176). RORA has been described to interact with cholesterol, 7-dehydrocholesterol and cholesterol sulfate (175, 177), while RORB and RORC have been found to bind several retinoids (178). However, it is not fully clear how these ligands modify DNA-binding capacity of ROR family members and whether ligand binding has a physiological relevance.

ROR family members interact with both co-activators and co-repressors to positively or negatively regulate transcription of target genes (Fig. 5). Together with co-activators they induce histone acetylation, leading to decompaction of the chromatin and therefore to activation of gene transcription (179-182). Interacting with co-repressors, ROR family members mediate chromatin de-acetylation, which is associated with gene repression (183-185).

ROR family transcription factors have been described to be involved in regulation of circadian rhythm and behavior (186-189). RORA is critically involved in the development of the retina by regulating several genes in the photoreceptors (190), but also in thymopoiesis and lymphocyte development (191) and in the lipid metabolism (192-196). RORB seems to

be important in the development of the brain and the retina, as RORB-deficient mice display a duck-like gait and retinal degeneration (187, 197). RORC plays a key role in the development of lymph nodes and Peyer's patches and in the regulation of thymopoiesis (170, 198-200).

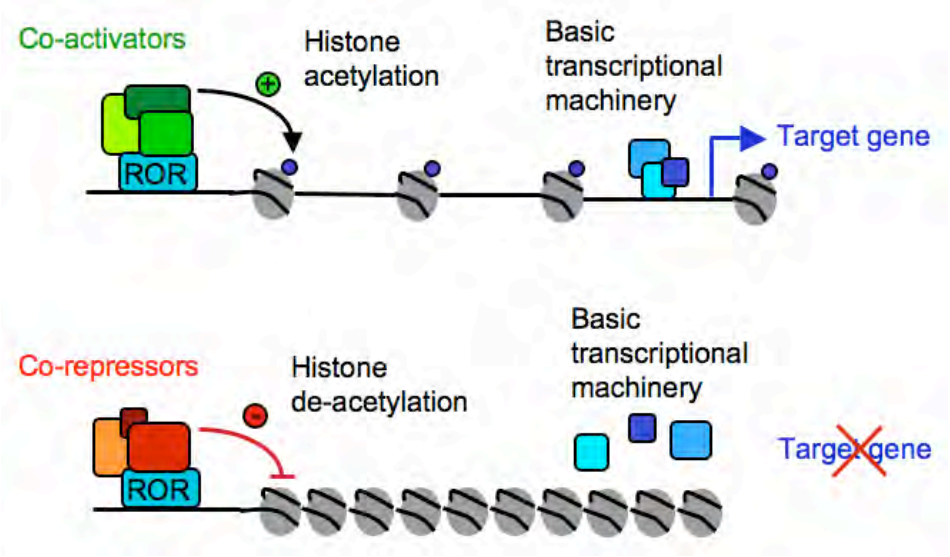

Fig 5. Transcriptional regulation by ROR family transcription factors. ROR family members bind to ROREs in the regulatory region of their target genes and interact with co-activators or co-repressors. Interaction with co-activators induces histone acetylation, which leads to decompaction of the chromatin and therefore to activation of gene transcription. Through interaction with co-repressors, ROR family transcription factors mediate chromatin de-acetylation, which is associated with gene repression.

1.3.4.1 Expression and function of RORC2

The two isoforms of RORC display very little differences in their amino acid sequence, but show a completely distinct expression pattern. While RORC1 is expressed in many tissues like liver, adipose tissue, skeletal muscle and kidney, RORC2 is expressed only in a few immune cells. Mice deficient in RORC2 lack lymph nodes and Peyer's patches, suggesting a crucial role for RORC2 in the development of certain lymphoid organs (199, 200). Failure in development of lymph nodes and Peyer's patches is due to an absence of lymphoid tissue inducer cells in RORC2-deficient mice (198). These cells play a critical role in the

development of lymph nodes and Peyer's patches, but also in the organogenesis of isolated lymphoid follicles, which are lymphoid compartments located in the intestines (201). In contrast, development of several other lymphoid tissues like nasal- and bronchial-associated lymphoid tissue (NALT and BALT) is not affected in RORC2-deficient mice, suggesting that development of these organs is governed by a different regulator (202).

Several studies suggested RORC2 to play a central role in thymopoiesis by regulating proliferation and apoptosis in immature thymocytes (170, 171, 199, 200, 203, 204). RORC2 positively regulates the anti-apoptotic gene *Bcl-X_L*, thereby promoting thymocyte survival. Thus, RORC2-deficient mice have a significantly smaller thymus in early life, due to a dramatically reduced number of thymocytes.

More recently, a role for RORC2 in T cell differentiation has been described and RORC2 is now accepted as key transcription factor of Th17 cells (153, 162, 163). RORC2-deficient mice show markedly reduced numbers of Th17 cells (153), and overexpression of RORC2 greatly increased IL-17 production (162, 163). RORC2 has been shown to bind to two RORE in an enhancer element of the *il17-il17f* locus, suggesting that RORC2 directly activates expression of these Th17-specific genes (167).

1.4 Objective

Th17 cells constitute an important part of the immune system's host defense. If not properly controlled, they induce or contribute to inflammation in many autoimmune disorders as well as in allergy and asthma. Treg cells, in contrast, suppress excessive immune responses, but they can undesirably interfere with immunity against in infections or cancer. Differentiation of naïve T helper cells towards one of these subsets must therefore be tightly controlled to establish equilibrium between protective immune responses and tolerance against harmless (self-) antigens. Understanding the mechanisms regulating this equilibrium is of great interest in order to develop therapies for autoimmune diseases.

The ~~aim~~ of this thesis was to gain insight into the pathways leading to the differentiation of Th17 cells and to analyze the mechanisms controlling the delicate balance between Th17 and Treg cell differentiation.

2 RESULTS

2.1 Differentiation and functional analysis of human T helper 17 cells

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Abstract

Background: T helper (Th) 17 cells are of pathologic relevance in autoimmune disorders and presumably also in allergy and asthma. Regulatory T (T_{reg}) cells, in contrast, suppress inflammatory and allergen-driven responses. Despite these disparate functions, both T cells subsets have been shown to be dependent on TGF-β for their development.

Objective: The aim of the study was to analyze differentiation and function of human Th17 cells in comparison with other T helper cell subsets.

Methods: Naïve human $CD4^+$ T cells were *in vitro*-differentiated and gene expression was analyzed by quantitative real-time PCR, ELISA and immunofluorescence. The function of T helper cell subsets was assed by monitoring the response of primary bronchial epithelial in co-culture experiments.

Results: *In vitro*-differentiated Th17 cells differ from T_{reg} and other T helper cells in their potency to induce IL-6 and IL-1β expression in primary bronchial epithelial cells. To acquire this functions including IL-17 production, TGF-β, IL-1β, IL-6 and IL-23 are necessary during Th17 cell differentiation. In contrast, TGF-β alone is necessary and sufficient to induce the transcription factor RORC2. This transcription factor, previously thought to be specific for Th17 cells, is also expressed in T_{reg} cells, $CD25^+$ cells, cytotoxic T cells and in natural killer T cells.

Conclusion: This study demonstrates mechanisms of differentiation to human Th17 cells, a subset that effectively and uniquely modulate function of primary bronchial epithelial cells.

Key Messages: Human Th17 cells differ from other subsets in their potency to induce proinflammatory cytokines in bronchial epithelial cells. The transcription factor RORC2 is not exclusively expressed in Th17 cells.

Capsule summary: Human Th17 cells represent a functionally distinct subset that potently induces proinflammatory cytokines in primary bronchial epithelial cells, suggesting an implication in airway inflammation.

Key words: Interleukin-17, regulatory T cells, differentiation, primary bronchial epithelial cells, airway inflammation, transcription factor, transforming growth factor β

Introduction

T helper cells translate antigen stimulation to tissues and instruct tissue cells to raise immune responses (205). The type of response depends on the properties of the T cells that interact with the tissue. In allergen-induced reactions, tissue cells are modulated by T helper (Th) 2 cells, which leads to a response including eosinophilia, IgE production, mucus hypersecretion and airway remodeling (206). Beside Th2 cells, also Th1, regulatory T (T_{reg}) and Th17 cells play a role in atopic diseases (205). Differentiation from naïve T cells towards distinct T helper subsets depends on the cytokine-milieu during differentiation and involves the lineage-specific transcription factors T-bet, GATA3 and FOXP3 for Th1, Th2, and T_{reg} cells, respectively. The retinoic acid receptor (RAR)-related orphan nuclear receptor gt (RORγt) has been shown to be necessary and sufficient for murine Th17 development (153). RORγt and its isoform RORγ belong to the ROR-subfamily of nuclear receptors, which also includes RORα and RORβ. Recently, a role for RORα in Th17 development has been described in mice (167).

Each T helper cell subset secretes a characteristic set of cytokines. IL-17A is specifically produced by the Th17 subset and coordinates tissue inflammation through induction of pro-inflammatory cytokines and chemokines like IL-6, TNF-α and IL-8 (207). Consistently, Th17 cells were shown to play a key role in many autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematous and inflammatory bowel disease (208-211). Moreover, there are studies suggesting that Th17 cells are also implicated in allergy and asthma (111, 212, 213). As a consequence, current research efforts attempt to elucidate the development and function of this pathogenic T cell population. Whereas the factors responsible for human Th17 cell development are still not completely clear, three independent studies describe a crucial role for TGF-β and IL-6 in the differentiation of murine Th17 cells (150-152). TNF-α and IL-1β amplify this process, while IL-23 is important for stabilization of already differentiated Th17 populations and for an effective pathogen defense (96, 214). Beside its role in the differentiation of Th17 cells, TGF-β is a crucial factor for the development of T_{reg} cells. It has been shown that TGF-β converts $CD4^+CD25^-$ T cells to T cells with a regulatory phenotype *in vitro* (4, 215, 216). However, the mechanisms downstream of TGF-β leading to such completely different T cell phenotypes are still not very well understood.

The current study investigates developmental pathways and effector function of human Th17 cells in relation to other T cell subsets and examines the role of TGF-β and other Th17-differentiating cytokines in these events. TGF-β is necessary and sufficient for the induction of the transcription factor RORC2, which we detect not only in Th17 cells but also in T_{reg} cells, $CD25^{+}$ cells, cytotoxic T cells and in natural killer T cells. In addition to TGF-β, also IL-1β, IL-6 and IL-23 are required for the differentiation of a Th17 population that potently induces proinflammatory cytokines in primary human bronchial epithelial cells.

Methods

Cell isolation

Peripheral blood mononuclear cells (PBMC) are isolated from buffy coats of healthy donors using Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation. $CD4^+$ T cells are purified with anti-CD4 magnetic beads and Detach-a-Bead antibodies (Dynal, Hamburg, Germany). $CD45RA^+$, $CD45RA^-$, $CD14^+$, $CD19^+$, $CD3^+CD56^+$, $CD25^-$, $CD25^+$ and $CD8^+$ cells are isolated using MACS magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For the purification of $CD3^+CD56^+CD25^-CD69^-$ cells, $CD56^+$ cells are isolated from PBMC using anti-CD56-PE antibodies (BD Bioscience, Allschwil, Switzerland) and anti-PE MACS magnetic beads (Miltenyi). Afterwards, $CD56^+$ cells are stained with anti-CD3-FITC, anti-CD25-PE-Cy5 and anti-CD69-PE-Cy5 antibodies (all Beckman Coulter, Krefeld, Germany) and $CD3^+CD56^+CD25^-CD69^-$ are sorted using a FACSAria II sorting system (BD Bioscience). Purity is higher than 97%.

Cell cultures

All cell cultures are carried out in serum-free AIM-V medium (Life Technologies, Basel, Switzerland) supplemented with IL-2 (25 U/mL, Proreo Pharma AG, Liestal Switzerland). $CD4^+CD25^-$, $CD4^+CD25^+$, $CD8^+$, $CD3^+CD56^+$ and $CD3^+CD56^+CD25^-CD69^-$ cells are stimulated for 12 h with soluble anti-CD3 (4 µg/mL) and anti-CD28 (4 µg/mL) antibodies. Anti-CD3 and anti-CD28 antibodies are produced using the hybridoma cell line OKT3 (American Type Tissue Collection, Manassas, VA, USA) and the hybridoma cell line 15E8 (CLB, Amsterdam, The Netherlands). $CD19^+$ cells are stimulated with soluble CD40 ligand (20 mg/ml, Biogen, Zug, Switzerland). $CD14^+$ cells are stimulated with lipopolysaccharide (LPS, 10 ng/ml, Sigma, Buchs, Switzerland). Neutralizing anti-TGF-β antibody is purchased from R&D (Abingdon, UK). IL-21 is purchased from eBioscience (San Diego, USA) and used at a concentration of 25 ng/ml)

Suppression assay

$CD4^+CD45RA^+$ T cells are cultured under T_{reg} condition during 10 days. At that point, autologous $CD4^+$ T cells are isolated. $CD4^+$ responder T cells are washed twice with PBS and labeled with 2 µM CFSE (Invitrogen, Paisley, UK) for 3 min at RT. After washing with

complete RPMI (Life Technologies), the cells are cultured in 96 round-bottom plates in the presence of anti-CD3 antibodies (4 µg/ml) during 5 days. The proliferation of $CD4^+$ responder T cells is analyzed by flow cytometry.

Differentiation of Th1, Th2, T_{reg} and Th17 cells

$CD45RA^+$ T cells are stimulated with soluble anti-CD3 (4 µg/mL) and anti-CD28 antibodies (4 µg/mL). Th0 cells are generated in the presence of neutralizing anti-IL-4 antibodies (5 µg/mL, R&D, Abingdon, UK), anti-IL-12 antibodies (25 ng/mL, R&D) and anti-IFN-γ antibodies (1 µg/ml, R&D). Th1 cells are differentiated in the presence of IL-12 (5 mg/ml, R&D) and neutralizing anti-IL-4 antibodies. Th2 cells are generated in the presence of IL-4 (25 ng/mL, R&D) and neutralizing anti-IL-12 antibodies. T_{reg} cells are generated, as previously described (13), in the presence of TGF-β (5 ng/mL, R&D), neutralizing anti-IL-12 antibodies and neutralizing anti-IFN-γ antibodies. Th17 cells are differentiated in the presence of TGF-β, IL-6 (20 ng/mL, PeproTech EC, London, UK), IL-1β (10 ng/mL, PeproTech EC), IL-23 (10 ng/mL, eBioscience, San Diego, USA), neutralizing anti-IL-12 antibodies and neutralizing anti-IFN-γ antibodies. Dose titrations are performed and the optimal dose for each cytokine and antibody is determined in preliminary experiments.

T cell coculture with NHBE cells

Normal human bronchial epithelial (NHBE) cells (LONZA, Visp, Switzerland) are maintained in BEBM (LONZA) and are plated in a 24-well dish to be 90% confluent the day of the experiment. NHBE cells are used at passage numbers 2 or 4. T cells are differentiated as described above. At d 6, T cells are washed to remove the cytokines used for differentiation and were restimulated for 24 h with anti-CD3 and anti-CD28 antibodies. T cells are added to NHBE cells at a number of $5x10^5$ cells per well, or cultured alone. Alternatively, the supernatants of washed and restimulated T cells are added. To block transcription in T cells, the T cells are incubated with Actinomycin D (10 µg/ml, Invitrogen, Paisley, UK) during the last hour of restimulation. To block cytokine secretion, T cells are incubated with Brefeldin A (10 µg/mL, Sigma, Buchs Switzerland). The T cells are washed to remove Actinomycin D and Brefeldin A before co-culturing. After 6 h of coculture, NHBE cells and T cells are harvested for mRNA analysis. Supernatants for cytokine analysis by ELISA and cytometric bead array are collected after 48 h. For immunofluorescence

microscopy, NHBE cells grown on coverslips are cocultured with T cells for 30 h. Brefeldin A is added during the last 3 h to block cytokine secretion.

Isolation of RNA, cDNA synthesis and quantitative real-time PCR

Total RNA is isolated using the RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Reverse transcription is performed with TaqMan reverse transcription reagents (Fermentas, Nunningen, Switzerland) using random hexamer primers according to the manufacturer's protocol.

The PCR primers and probes are designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Applied Biosystems). Primers used for relative quantification are listed in table 1. All primers are spanning intron-exon borders and are verified for efficacy over a 4-log concentration range. The prepared cDNAs are amplified using iTaq SYBR Green Supermix with ROX (BioRad, Basel, Switzerland) according to the manufacturer's recommendations in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Relative quantification and calculation of the range of confidence is performed using the comparative $\Delta\Delta$CT method. EF-1α is used as endogenous control. All amplifications are conducted in triplicates.

Determination of cytokine concentration

T cells are restimulated at d 7 with anti-CD3 and anti-CD28 antibodies. After 48 h, supernatants are collected and IL-17A and IL-6 levels are analyzed by ELISA (eBioscience and PeproTech EC, respectively) according to the manufacturer's instructions. Concentrations of IFN-γ, IL-1β, IL-4, IL-5, IL-8, IL-13, IL-17A, G-CSF and GM-CSF are determined by cytometric bead array (BioRad, Reinach, Switzerland) according to the manufacturer's protocol.

Immunofluorescence

The cells grown on coverslips are fixed for 10 min with 1% paraformaldehyde (Fluka, Buchs, Switzerland) and permeabilized with detergent (0.1% Triton (Fluka), 0.02% SDS (Roth, Karlsruhe, Germany) in PBS (Invitrogen, Basel, Switzerland) for 5 min. Next, the cells are blocked in 2% BSA (Fluka) in PBS for 30 min, probed for 1 h with fluorescein isothiocyanate (FITC)-labeled anti-IL-6 antibodies (1:5, BioLegend, Uithoorn, The Netherlands) and again

fixed in 1% paraformaldehyde. After mounting using VectaShield mounting medium containing 4',6-Diamidino-2-phenylindol (DAPI) (Vector Laboratories, Burlingame, CA), the cells are analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems AG, Glattbrugg, Switzerland).

Table 1. Primers used for quantitative real-time PCR

EF-1α fw	CTGAACCATCCAGGCCAAAT
EF-1α rv	GCCGTGTGGCAATCCAAT
FOXP3 fw	GAAACAGCACATTCCCAGAGTTC
FOXP3 rv	ATGGCCCAGCGGATGAG
GATA3 fw	GCGGGCTCTATCACAAAATGA
GATA3 rv	GCTCTCCTGGCTGCAGACAGC
IL-1β fw	GCAATGAGGATGACTTGTTCTTTG
IL-1β rv	CAGAGGTCCAGGTCCTGGAA
IL-6 fw	GGTACATCCTCGACGGCATCT
IL-6 rv	AGTGCCTCTTTGCTGCTTTCAC
IL-17A fw	CCATCCCCAGTTGATTGGAA
IL-17A rv	CTCAGCAGCAGTAGCAGTGACA
IL-17F fw	CAGCGCAACATGACAGTGAA
IL-17F rv	CCAATATCGACAGCAGCAAGTACT
RORA fw	TGAGAGAAGAGCTCCAGCAGATAAC
RORA rv	CACCTCCCGCTGCTTGTTT
RORA4 fw	GCAGCGATGAAAGCTCAAATT
RORA4 rv	CCCTTGCAGCCTTCACATGT
RORB fw	TTTCAACAATGGGCAGTTAGCA
RORB rv	CCAAATGGGACTTAATGATGTTCTG
RORC1 fw	GACAGGGCCCCACAGAGA
RORC1 rv	TTTGTGAGGTGTGGGTCTTCTTT
RORC2 fw	CAGTCATGAGAACACAAATTGAAGTG
RORC2 rv	CAGGTGATAACCCCGTAGTGGAT
T-bet fw	GATGCGCCAGGAAGTTTCAT
T-bet rv	GCACAATCATCTGGGTCACATT

Results

IL-17 is regulated by TGF-β, IL-1β, IL-6 and IL-23

Understanding the specific functions of T helper cell subsets is important for the comprehension of immune regulation and pathological mechanisms. We investigated whether *in vitro*-differentiated Th17 cells differ from other T cell subsets regarding their effector function. As the factors required for the differentiation of human Th17 cells are still under debate, we first tested several cytokines for their ability to induce Th17-specific genes in naïve human T cells. Naïve T cells were stimulated for 7 d with IL-1β, IL-6, IL-21 and IL-23 or combinations in the presence or absence of TGF-β (Fig. 6A). The highest induction of IL-17A and IL-17F mRNA is observed in cells cultured with IL-1β, IL-6, IL-23 and TGF-β, with or without IL-21. Database search revealed that RORC2 is the closest human homologue of mouse RORγt (mRORγt), therefore we included RORC2 in the analysis. High RORC2 mRNA levels are observed in cells treated with TGF-β, whereas the other cytokines tested have a minor effect on RORC2 levels.

Similar to the IL-17A and IL-17F mRNA data are the results observed for IL-17A protein secretion (Fig. 6B). The highest amounts of IL-17A are produced by cells stimulated with TGF-β, IL-1β, IL-6, IL-21 and IL-23 (11.9) ng/ml). Interestingly, omission of IL-21 in this cytokine cocktail resulted in nearly the same amount of IL-17A (11.5 ng/ml). Taken together, these results suggest that TGF-β is sufficient for RORC2 induction, while IL-1β, IL-6 and IL-23, and to a lesser extend IL-21, are required for production of IL-17A and IL-17F.

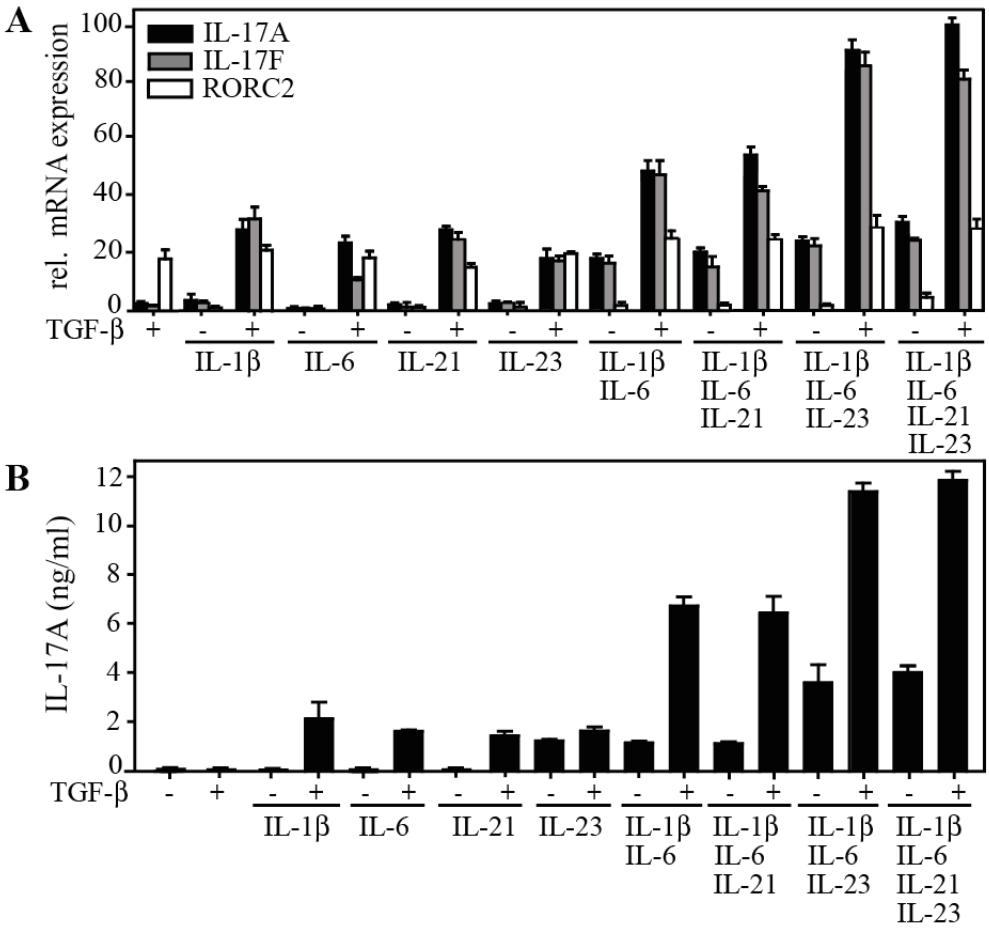

Fig 6. Regulation of Th17-related genes IL-17A, IL-17F and RORC2. Naïve T cells were stimulated for 7d with the cytokines indicated. **A)** After 6h of restimulation, IL-17A, IL-17F and RORC2 levels were measured by real-time PCR and normalized to levels in untreated cells. Mean and standard deviations of triplicates are shown. **B)** After 48h of restimulation, IL-17A secretion was determined by ELISA. n.d. = not detectable. Mean and standard errors of duplicates are shown. Data are representative for at least 3 independent experiments.

Human in vitro-differentiated Th17 cells stimulate IL-6 and IL-1β production in primary bronchial epithelial cells

Next, we wanted to investigate, whether *in vitro*-differentiated Th17 are a functionally distinct T helper cell subset. As IL-17A is known to induce IL-6 in several tissue cells (207), we analyzed IL-6 production by normal human bronchial epithelial (NHBE) cells, a primary cell line, upon stimulation with Th17 cells. Naïve T cells were cultured for 7 d under conditions driving Th1, Th2, T_{reg} and Th17 differentiation (see materials and methods section), or without cytokine addition (designated as Th0). After 7 d of differentiation, T cells

were washed to remove the differentiation cytokines and were restimulated. To test the efficiency of the differentiation process, cytokine profiles of T cells subsets were verified by cytometric bead array (data not shown). The function of T_{reg} cells was initially assed by suppression assays. T cells were added to NHBE cells and expression of IL-6 was measured by real-time PCR (Fig. 7A) and ELISA (Fig. 7B). While NHBE cells cocultured with Th2 and T_{reg} cells show only slightly increased IL-6 production compared to cells cultured alone, NHBE cells cultured in the presence of Th17 cells produce about 20-times more IL-6 (3.45±0.5 ng). Th1 and Th0 cells also stimulate IL-6 production in NHBE cells, but to a lesser extent. Some IL-6 is detected in Th17 cells, but not in other T cell subsets. Of note, IL-6 production by NHBE cells also substantially increases when the cells are stimulated with cell-free supernatants of Th17 cell cultures (Fig. 8A). This observation suggests that cell-cell contacts are not necessary for the induction of IL-6. Interestingly, Th17 cells co-cultured with NHBE cells produce almost double the amount of IL-17A than Th17 cells cultured alone, suggesting a positive feedback loop generated by NHBE cells (Fig. 8B). Even though the T cells were washed before co-culturing with epithelial cells, carry-over of the differentiation cytokines to the coculture may occur, for example via receptors on the cell surface. To exclude that these cytokines account for the observed IL-6 induction, we analyzed, whether *de novo* protein secretion by T cells is required to induce IL-6. Therefore, protein secretion in T cells was abolished by preincubating the T cells with Actinomycin D, which inhibits transcription, or Brefeldin A, which blocks cytokine secretion (Fig. 8C and D). T cells pre-treated with these substances are no longer able to induce IL-6 production in epithelial cells, showing that production and secretion of cytokines by T cells is needed and the IL-6 induction is not due to a carry-over of the differentiation cytokines.

To further confirm that Th17 and T_{reg} cells differ in their modulation of NHBE cells regarding IL-6 production, bronchial epithelial cells cultured with Th17 cells and T_{reg} cells were stained for IL-6 and analyzed by confocal microscopy (Fig. 7C). In contrast to NHBE-T_{reg} cocultures, there is a clear IL-6 cytokine staining in most of the NHBE cells cultured in the presence of Th17 cells, consistent with our results obtained by real-time PCR and ELISA.

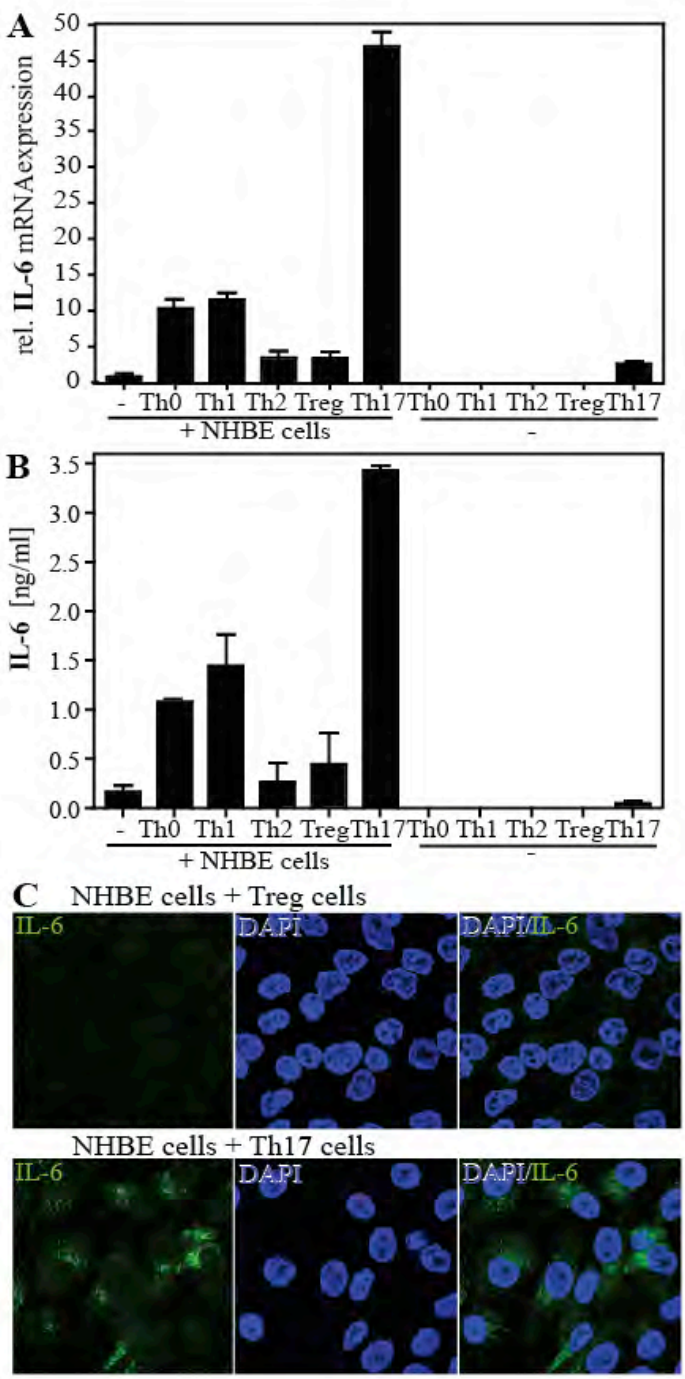

Fig 7. Th17 cells stimulate IL-6 production by bronchial epithelial cells. NHBE cells were cultured alone or in presence of T cells. **A)** After 6 h, IL-6 mRNA was quantified by real-time PCR and normalized to levels in NHBE cells cultured alone. Mean and standard deviations of triplicates are shown. **B)** IL-6 was measured by ELISA after 48 h of coculture. Mean and standard errors of duplicates are shown. **C)** NHBE cells were cultured in presence of T_{reg} or Th17 cells. After 30 h, NHBE cells were stained with DAPI and anti-IL-6 antibodies and analyzed by confocal microscopy. Data shown are representative for 3 independent experiments.

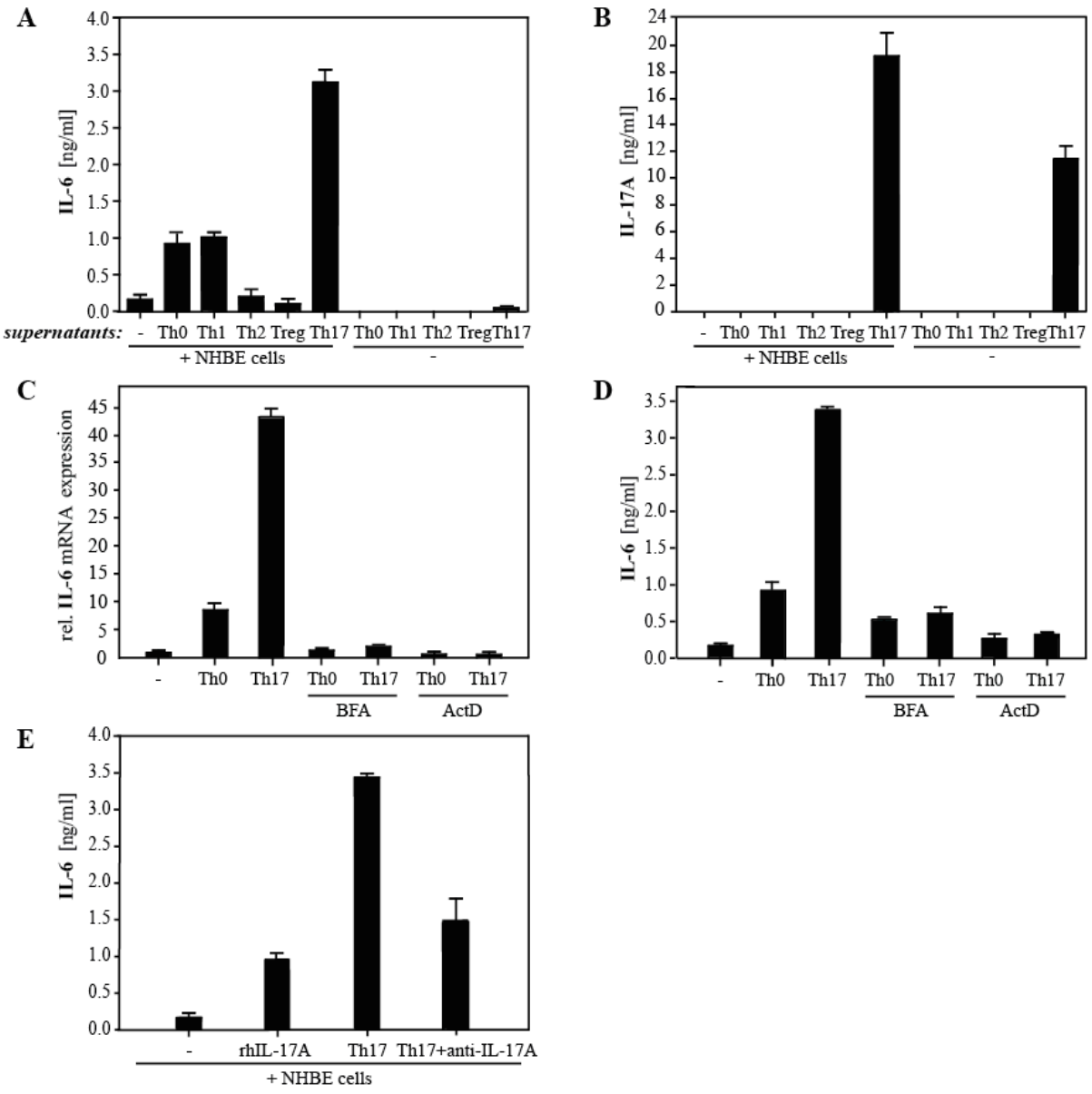

Fig 8. Regulation of IL-6 and IL-17A expression in T cells and NHBE cells. A) NHBE cells were cultured for 48 h with supernatants from different T cell subsets. IL-6 protein secretion was measured by ELISA. **B)** NHBE cells were cultured with T cells differentiated to different subsets. After 48 h of coculture, IL-17A protein secretion was measured by ELISA. **C)** T cells differentiated to Th0 and Th17 cells were treated for 1 h with Brefeldin A (BFA) or Actinomycin D (ActD), or left untreated. After washing, the T cells were cocultured with NHBE cell for 6 h to analyze IL-6 expression by real-time PCR (**C**) or for 48 h to measure IL-6 protein by ELISA (**D**). **E)** NHBE cells were cultured for 48 h with recombinant IL-17A (rhIL-17A) or with Th17 cells in the presence or absence of neutralizing anti-IL-17A antibodies. IL-6 protein secretion was measured by ELISA. Mean and standard errors of duplicates are shown. Data are representative for at least 3 independent experiments.

Beside IL-6, we find IL-1β to be highly increased in NHBE-Th17 cocultures compared to NHBE cells cultured alone or together with T_{reg} cells (Fig. 9A and 9B, upper left panel). Furthermore, increased levels of IL-8, G-CSF and GM-CSF are observed in NHBE cells cocultured with Th17 cells (Fig. 9B).

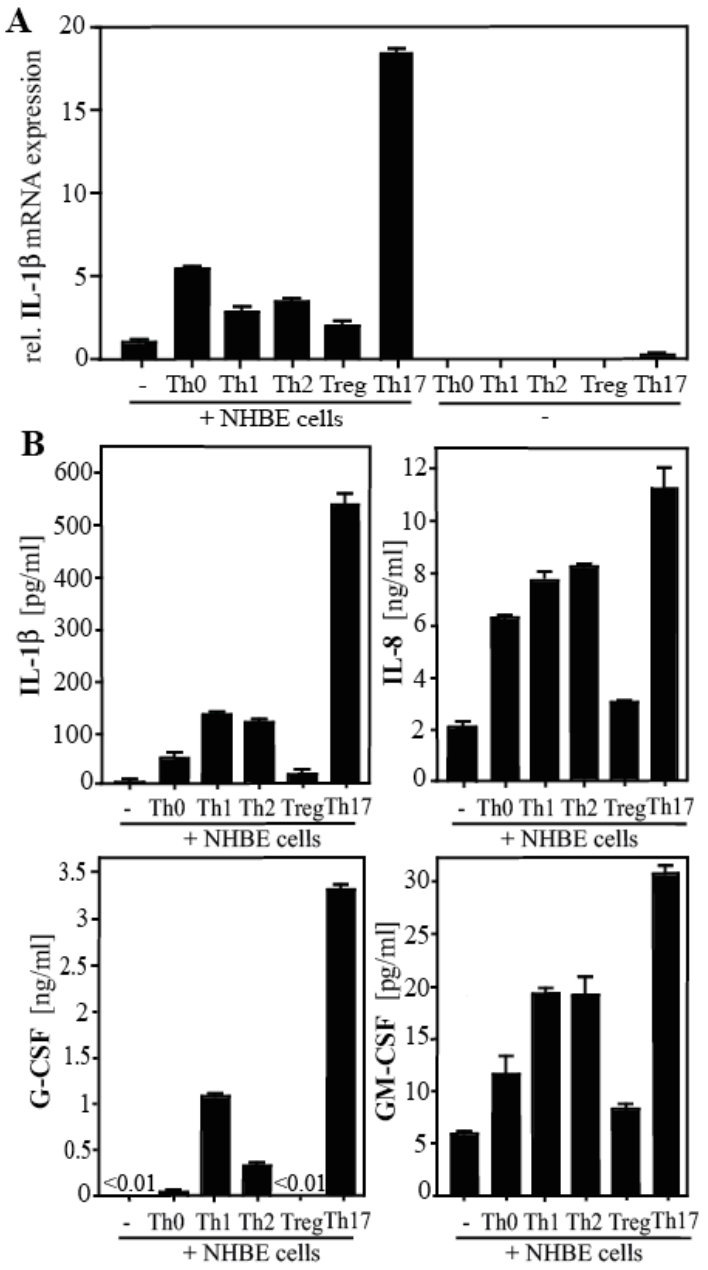

Fig 9. Th17 cells stimulate cytokine and chemokine production by bronchial epithelial cells. NHBE cells were cocultured with T cells. **A)** After 6 h, IL-1β mRNA levels were measured by real-time PCR and normalized to levels in NHBE cells cultured alone. Mean and standard deviations of triplicates are shown. **B)**. After 48 h, IL-1β, IL-8, G-CSF and GM-CSF concentrations were determined by cytometric bead array. Mean and standard errors of duplicates are shown. Data are representative for 3 independent experiments.

Taken together, these findings show that human *in vitro*-differentiated Th17 cells make up a distinct effector subset, which substantially differs from other T helper cells subsets in its ability to induce production of pro-inflammatory cytokines by primary bronchial epithelial cells.

RORC2 is highly expressed in Th17 cells and in cells differentiated under T_{reg} conditions

The human ROR-subfamily consists of three members: RORA, RORB and RORC (Fig. 10A). Human RORC (hRORC) as well as mouse RORγ (mRORγ) exist in two isoforms. mRORγ and its isoform mRORγt are identical from exon 3 to 11, while exon 2 is missing in mRORγt. Each of the isoforms contains a distinct exon 1. The same pattern is observed for hRORC1 and hRORC2. hRORC2 shows the same exon configuration as mRORγt, while hRORC1 corresponds to mRORγ.

The sequence homology of mRORγt and hRORC2 as well as two recent publications suggest RORC2 to be the key transcription factor for differentiation towards human Th17 cells (162, 163). To investigate whether RORC2 is selectively expressed in the Th17 subset of T helper cells, naïve T cells were differentiated under Th1, Th2, T_{reg} or Th17 conditions and expression of their lineage-specific transcription factors was measured (Fig. 10B). Interestingly, high levels of RORC2 mRNA are found not only in Th17, but also in T_{reg} cells, while Th1 and Th2 cells show only low RORC2 expression. T-bet, GATA3 and FOXP3, on the other hand, are specifically expressed in Th1, Th2 and T_{reg} cells, respectively, showing that differentiation generated distinct T helper cell populations.

Recently, RORα, specifically isoform 4, has been suggested to play an important role in murine Th17 development (167). To test whether RORA, the human homologue of mouse RORα, is specifically expressed in Th17 cells, Th1, Th2, T_{reg} and Th17 cells were analyzed for the expression of RORA (all isoforms), RORA4 (isoform 4) and RORC2 (Fig. 10C). While expression of RORA and RORA4 is slightly higher in Th17 and T_{reg} cells, the differences to levels in Th1 and Th2 are not as clear as for RORC2. Next, we analyzed the expression of RORC2 in different leukocytes. Monocytes ($CD14^+$), B cells ($CD19^+$), natural killer (NK) T cells ($CD3^+CD56^+$), $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells and cytotoxic T cells ($CD8^+$) were stimulated with and without TGF-β and are analyzed for RORC2 and IL-17A mRNA expression (Fig. 10D). While RORC2 expression is low in $CD14^+$ and $CD19^+$ cells,

high levels of RORC2 are found in $CD3^+CD56^+$ and $CD8^+$ cells. Both $CD4^+CD25^-$ and $CD4^+CD25^+$ cells highly express RORC2, whereas IL-17A, in contrast, is high only in $CD4^+CD25^-$ cells, but cannot be detected in $CD4^+CD25^+$ cells, consistent with the suppressive role of these cells. Given the high expression of RORC2 in NKT cells, isolated using the surface markers CD3 and CD56, we wanted to exclude the possibility that contaminating activated T cells, which also express CD3 and CD56, are the source of RORC2. Therefore, we depleted activated T cells from NKT cells according to the activation markers CD25 and CD69 (Fig. 11A). PMBC were stained with antibodies against CD3, CD56, CD25 and CD69 and the $CD3^+CD56^+CD25^-CD69^-$ population was isolated by FACS sorting. Real-time PCR analysis of these cells revealed a RORC2 expression similar to the expression detected in $CD3^+CD56^+$ cells, indicating that activated T cells are not the main source of the high RORC2 levels observed (Fig. 11B).

In conclusion, our findings show that neither RORC2 nor RORA are likely to be specific markers for human Th17 cells, as both are also highly expressed under T_{reg} conditions and do not necessarily implicate IL-17 expression.

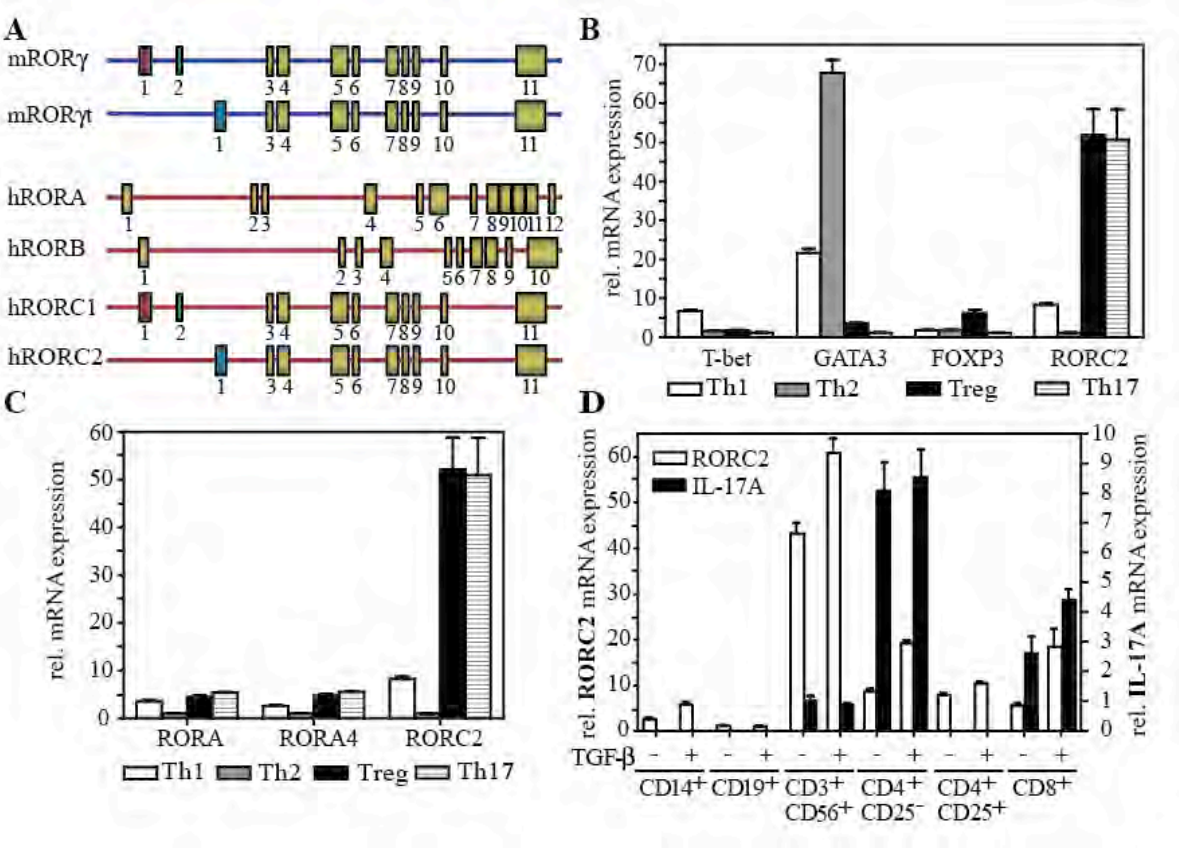

Fig 10. Expression of ROR-family members in T cell and leukocyte subsets. A) Schematic depiction of mouse and human ROR-family members. hRORC2 shares its exon configuration with mRORγt, while hRORC1 corresponds to mRORγ. hRORA is the homologue of mRORα. **B)** T cell subsets were analyzed for the expression of T-bet, GATA3, FOXP3 and RORC2. Lowest expression level of each transcription factor was set to 1. **C)** T cell subsets were analyzed for RORA, RORA4 and RORC2 expression. Primers recognizing all isoforms of RORA (RORA) and primers recognizing specifically isoform 4 (RORA4) were used for real-time PCR. Lowest expression level of each transcription factor was set to 1. **D)** Different leukocyte subsets were stimulated for 12 h with or without TGF-β. RORC2 (left scale) and IL-17A (right scale) mRNA levels were determined by real-time PCR. Error bars represent standard deviations of triplicates. Data are representative for at least 3 independent experiments.

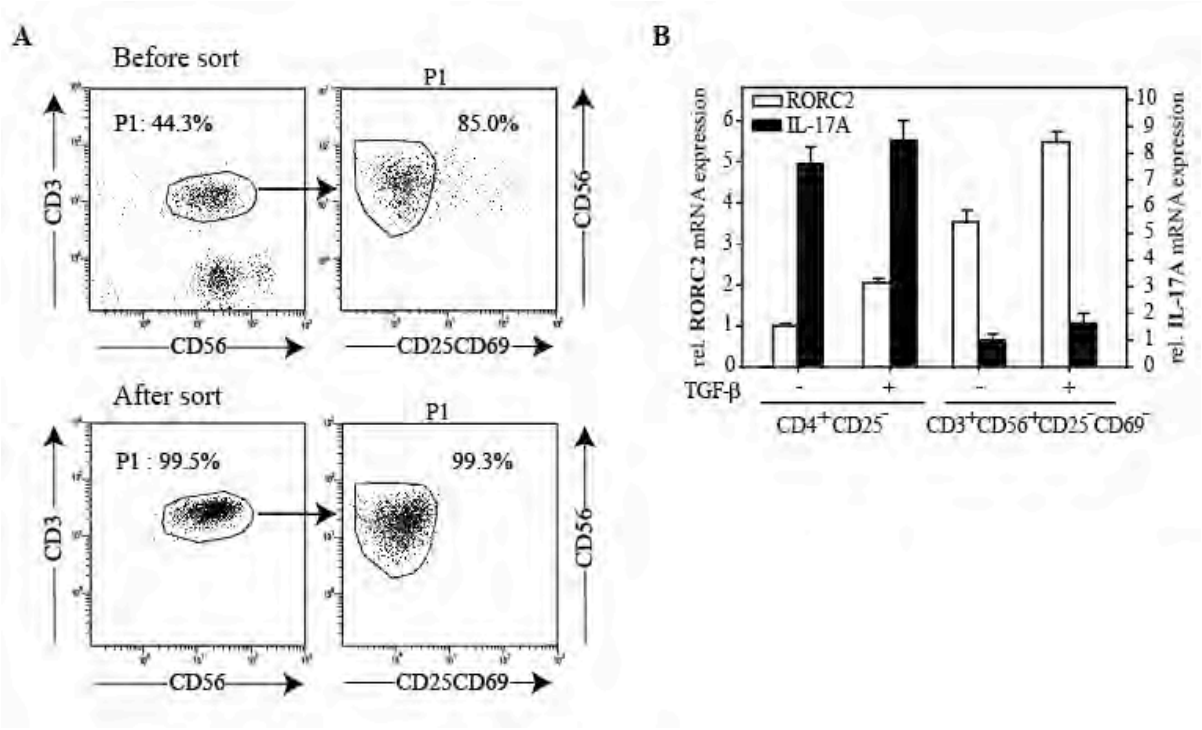

Fig 11. RORC2 expression in CD3$^+$CD56$^+$CD25$^-$CD69$^-$ cells. A) PMBC were stained with antibodies against CD3, CD56, CD25 and CD69 and the CD3$^+$CD56$^+$CD25$^-$CD69$^-$ population was isolated by FACS. **B)** After stimulation for 12 h with or without TGF-β, RORC2 (left scale) and IL-17A (right scale) mRNA levels were determined by real-time PCR. Error bars represent standard deviations of triplicates. Data are representative for 3 independent experiments.

TGF-β is necessary and sufficient to induce RORC2 in human T cells

As RORC2 is highly expressed in conditions with TGF-β (Fig. 6A), we asked the question, whether TGF-β regulates its expression. Naïve T cells were stimulated with anti-CD3 and anti-CD28 antibodies and a dose-titration of TGF-β was performed (Fig. 12A and 12B). While RORC2 is dose-dependently induced by TGF-β (Fig. 12A), IL-17A and IL-17F show a maximal expression at a concentration of 5 ng/ml (Fig. 12B). This concentration was previously found to be optimal for IL-17A and IL-17F expression when combined with other Th17-differentiating cytokines (data not shown). To further confirm these data and to see, whether TGF-β is required for RORC2 expression, we cultured naïve T cells for 7 d under Th17 conditions in the presence of neutralizing anti-TGF-β antibodies and measured RORC2 expression by real-time PCR (Fig 12C). Consistent with its dose-dependent induction by

TGF-β, RORC2 expression decreases with increasing concentrations of neutralizing antibodies. Taken together, these results suggest a strong dependency of RORC2 on TGF-β.

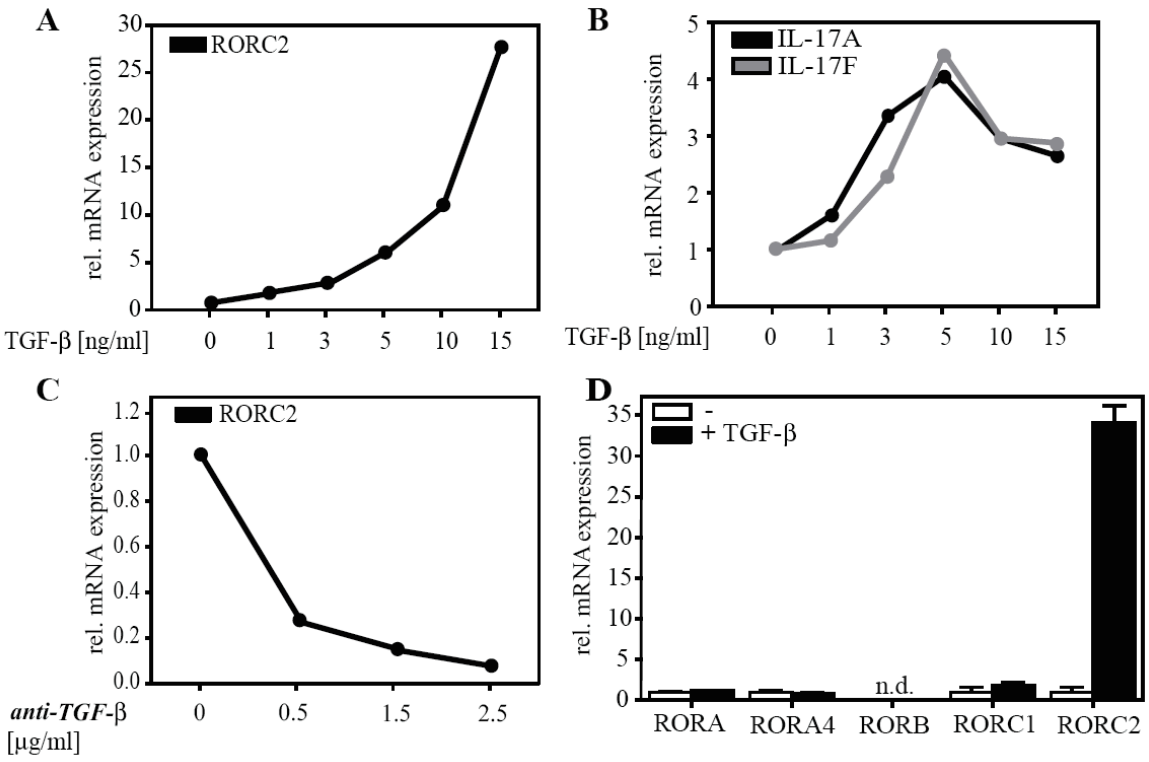

Fig 12. Effect of TGF-β on IL-17A, IL-17F and ROR-family members. Naïve T cells were stimulated with anti-CD3 and anti-CD28 antibodies and a TGF-β dose-titration was performed. At d 7, mRNA levels of RORC2 (**A**), IL-17A (**B**) and IL-17F (**B**) were measured by real-time PCR. **C)** Naïve T cells were stimulated under Th17 conditions together with neutralizing anti-TGF-β antibodies. At d 7, RORC2 levels were measured. **D)** Naïve T cells were stimulated with or without TGF-β and expression of RORA, RORA4, RORB, RORC1 and RORC2 was measured by real-time PCR. n.d.: not detectable. Error bars represent standard deviations of triplicates. Data are representative for at least 3 independent experiments.

A short-term kinetic (Fig 13A) and a long-term kinetic (Fig 13B) of RORC2 were performed. While RORC2 levels continuously decrease in the absence of TGF-β, there is a first peak of RORC2 expression between 9 h and 12 h if TGF-β is added in the beginning of the culture. A second, higher peak is observed at day three, which might reflect the expansion of a Th17 and/or T_{reg} population. At this time-point, the difference between cells stimulated with and

without TGF-β is maximal. The highest RORC2 expression is observed when cells, initially treated with TGF-β, are restimulated at day 7.

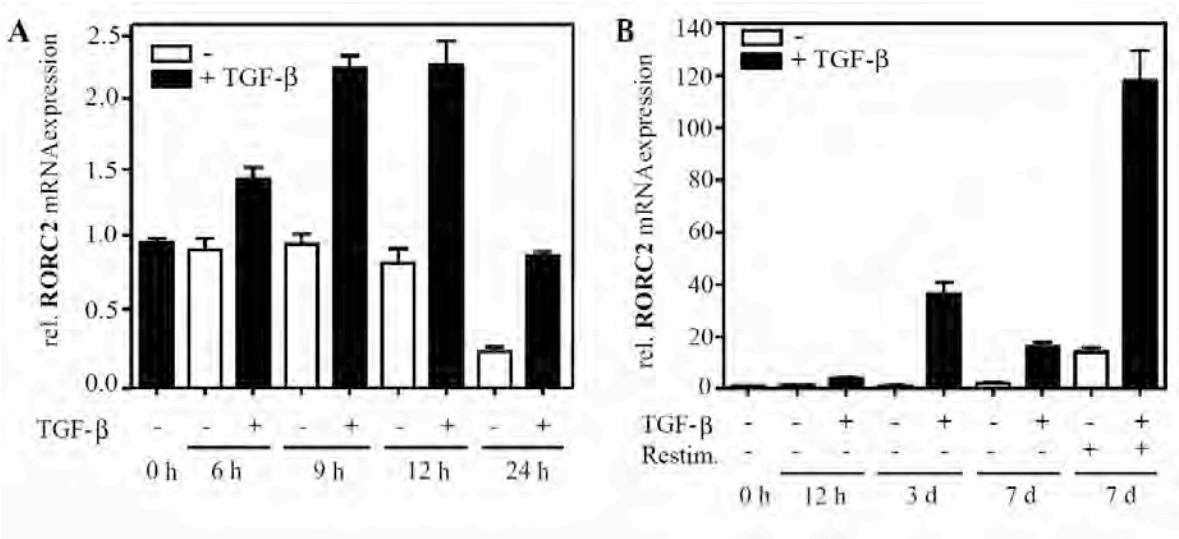

Fig 13. Time-kinetic of RORC2 expression. $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies and treated with (black) or without (white) TGF-β. RORC2 mRNA expression was determined by real-time PCR after 0 h, 6 h, 9 h, 12 h and 24 h (**A**), and after 0 h, 12 h, 3 d and 7 d, with and without restimulation at d 7. Levels in *ex vivo* samples (0 h) were set to 1. (**B**). Mean and standard deviations of triplicates are shown. Data are representative for at least 3 independent experiments.

To test whether the dependency on TGF-β applies also for the other ROR-family members, cells were stimulated for 7 d with or without TGF-β and mRNA levels of RORA (all isoforms), RORA4 (isoform 4), RORB, RORC1 and RORC2 were determined (Fig 12D). Of all genes tested, only RORC2 increases upon stimulation by TGF-β, fitting to its role in Th17 cell differentiation, which is dependent on TGF-β.

Next, we wanted to investigate if other Th17-differentiating cytokines affect RORC2 gene regulation. Therefore, naïve T cells were cultured for 7 d with IL-6, IL-1β or IL-23 in the presence or absence of TGF-β (Fig. 14A). None of these cytokines, except TGF-β, upregulates RORC2. Furthermore, combining the cytokines with TGF-β does not lead to a significant higher RORC2 expression compared to induction by TGF-β alone. This shows that of all cytokines important for Th17 differentiation, only TGF-β upregulates RORC2. Also other cytokines like IL-2, IL-4, IFN-γ, IL-10, IL-21, IL-27, TNF-α, or the lipidic mediator

retinoic acid (RA), do not upregulate RORC2 mRNA (Fig. 14B). Instead, IL-4 and RA, and to a lesser extent also IFN-γ, IL-10 and IL-27, reduce the TGF-β-mediated RORC2 induction. In summary, our results show that *in vitro*-differentiated Th17 cells make up a distinct effector subset as they differ in their potency to induce IL-6 and IL-1β in NHBE cells. TGF-β is necessary and sufficient for the induction of RORC2, which is expressed under Th17 but also under T_{reg} conditions. In contrast, IL-1β, IL-6 and IL-23 are required for the expression of IL-17A and IL-17F and for Th17-specific effector functions.

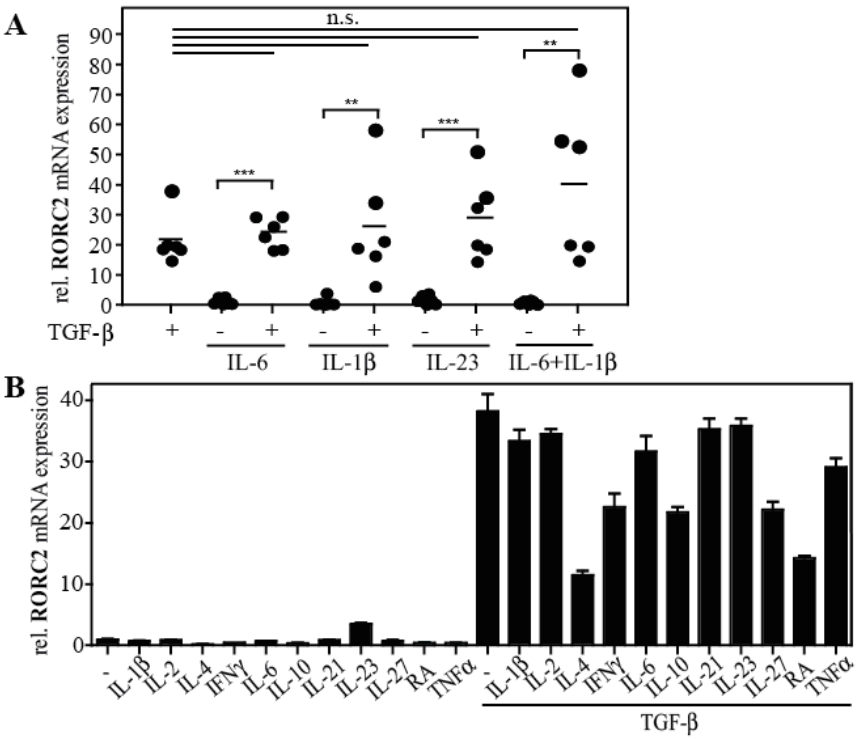

Fig 14. TGF-β is essential and sufficient for RORC2 induction. A) Naïve T cells were stimulated as indicated. At d 7, levels of RORC2 were measured. Statistical significance was tested using unpaired t tests (** $P \leq 0.01$, *** $P \leq 0.001$). **B)** Naïve T cells were stimulated for 7 d as indicated and RORC2 levels were determined. RA: Retinoic acid. Mean and standard deviations of triplicates are shown. Data are representative for at least 3 independent experiments.

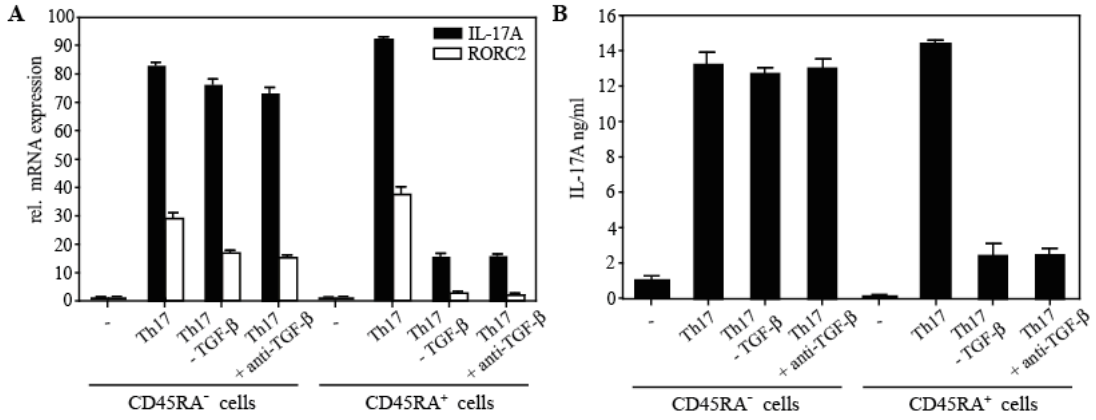

Fig 15. Effect of TGF-β on IL-17A and RORC2 in naïve and memory cells. Memory (CD45RA$^-$) and naïve (CD45RA$^+$) T cells were cultured under Th17 conditions in the presence or absence of TGF-β, or with neutralizing anti-TGF-β antibodies. **A)** Expression of IL-17A and RORC2 was measured by real-time PCR. Levels were normalized to levels in untreated cells of the corresponding population. Mean and standard deviations of triplicates are shown. **B)** IL-17A protein expression was measured by ELISA. Mean and standard errors of duplicates are shown. Data are representative of at least 3 independent experiments.

Discussion

This study demonstrates that human Th17 cells represent a functionally distinct subset of T helper cells that clearly differs from T_{reg} cells in its ability to modulate gene expression in primary bronchial epithelial cells, although both subsets share RORC2 expression.

Epithelial cells have been reported to produce IL-6 upon stimulation with recombinant IL-17A (207), and elevated levels of both IL-6 and IL-17A have been found in plasma and airways of asthmatic individuals (213, 217). Our data showing that *in vitro*-differentiated Th17 cells, in contrast to T_{reg} cells, potently induce IL-6 production by primary bronchial epithelial cells provide a further link between these cytokines and are a hint that Th17 cells contribute to airway inflammation in acute asthma. In addition, Th17 cells up-regulate G-CSF, GM-CSF and IL-8 production by bronchial epithelial cells, consistent with their role in granulopoiesis and attraction of neutrophils (143, 218). Th17 cells as well as supernatants from Th17 cell cultures induce higher levels of IL-6 than equal amounts of recombinant IL-17A, and this induction cannot be fully abrogated by anti-IL-17A antibodies (Fig. 8E). Therefore, we speculate that other Th17 cytokines such as IL-22, IL-26 or IL-17F, which have been described to regulate inflammatory responses (219), synergize with IL-17A in the induction of proinflammatory cytokines in epithelial cells. The fact that Th17 cells cocultured with bronchial epithelial cells produce more IL-17A than T cells cultured alone indicates that Th17 cells propagate the differentiation of their own population through induction of pro-inflammatory cytokines like IL-6 and IL-1β; a positive feedback mechanism observed for other Th subsets.

Th17 cells express the transcription factor RORC2, which has very recently been shown to play a crucial role in Th17 differentiation (162, 163). We observe high levels of RORC2 also in $CD8^+$ as well as in NKT cells, consistent with the expression of IL-17A in these cells (220-222). However, RORC2 is also expressed in cells differentiated towards T_{reg} cells, as well as in naturally occurring $CD25^+$ T_{reg} cells, which do not express IL-17A. This result suggests that the presence of RORC2 does not necessarily implicate IL-17A production. Expression of IL-17A in T_{reg} cells might be suppressed by FOXP3, which we detect selectively in T_{reg}, but not in Th17 cells. A hint for such a repression comes from two recent studies showing that Foxp3 antagonizes RORγt in mice (14, 15). These findings suggest that RORC2, although important for Th17 development, is not a selective marker for IL-17-producing cells and that

investigation of Th17 cells requires an assessment of additional characteristic genes such as IL-17A or IL-17F.

The factors required for the development of human Th17 cells are controversially discussed in literature. We found that TGF-β, the proinflammatory cytokines IL-1β and IL-6, as well as IL-23 are required for an efficient production of IL-17A and IL-17F and for the development of a functional Th17 effector population. A recent publication suggests a role for IL-21 in the development of human Th17 cells (164). Consistently, we observe a slight IL-17A induction by TGF-β and IL-21. However, the addition of IL-21 to the cytokine cocktail TGF-β, IL-1β, IL-6 and IL-23 does not lead to a further enhancement of IL-17A and IL17F. As Th17 cells have been described as a source of IL-21 (92), it might be that the cells cultured with TGF-β, IL-1β, IL-6 and/or IL-23 secrete substantial levels of IL-21, which then acts in an autocrine manner, overcoming an effect of supplemental IL-21.

The necessity for TGF-β in the development of human Th17 cells is currently under debate. In serum-free conditions we observe a crucial role for TGF-β in the induction of Th17-specific genes IL-17A, IL-17F and RORC2. The discrepancy in the question whether TGF-β is required or not might be due to differences in culture conditions such as usage of serum. It is anticipated that an appropriate concentration of TGF-β is crucial and that highly dosed TGF-β suppresses IL-17, as observed for many cytokines. Furthermore, TGF-β might be required at the beginning of the Th17 development, but at later stages rather inhibit IL-17 production. This is supported by our observation that neutralizing anti-TGF-β antibodies decrease IL-17 production in naïve T cells cultured under Th17 conditions, whereas these antibodies do not affect IL-17 production in memory cells (Fig. 15). Consistent with these results, a recent publication suggests that TGF-β is not required for IL-17 induction in memory cells (223).

Our data suggest that TGF-β contributes to the development of Th17 cells through induction of their transcription factor RORC2. None of the other cytokines important for Th17 cell development upregulates RORC2, nor do they synergize with TGF-β. Furthermore, TGF-β-induced RORC2 expression is inhibited by IL-4 and RA, which were reported to antagonize Th17 lineage commitment (2, 3, 60). The mechanisms underlying this inhibition, however, are still unclear. In conclusion, this study identifies a crucial role for TGF-β in the induction of the transcription factor RORC2. The fact that RORC2 expression does not always implicate IL-17 expression highlights the need for additional markers to identify Th17 cells in

disease. By comparing effector functions of different T helper cell subsets, we identify a strong capacity of human Th17 cells in the induction of highly proinflammatory cytokines in primary bronchial epithelial cells, suggesting a role for Th17 cells not only in autoimmune disorders, but also inflammatory airway diseases.

2.2 RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter

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Running title: RORC2 inhibits Tregs through interaction with the FOXP3 promoter

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Abbreviations: ATF: activating transcription factor; CREB: cAMP response element-binding protein; DBD: DNA-binding domain; FOXP3: forkhead box p3; GATA-3: GATA binding protein 3; LBD: ligand-binding domain; RAR: retinoic acid receptor; RORE: ROR-response element; RORC2: RAR-related orphan receptor C2; siRNA: small interfering RNA; T-bet: Th1-specific T box transcription factor; Treg: regulatory T; TSS: transcription start site

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Abstract

The process of T helper cell differentiation towards effector T cells is important to tailor specific immunity to invading pathogens, while tolerating harmless or autologous antigens. Identification of the mechanisms underlying this polarization process is therefore central to understand how the immune system confers immunity and tolerance. The present study demonstrates that RORC2, a key transcription factor in Th17 cell development, inhibits FOXP3 expression in human T cells. While over-expression of RORC2 in naïve T cells reduces levels of FOXP3, siRNA-mediated knockdown of RORC2 enhances its expression. RORC2 mediates this inhibition at least partially by binding to two out of four ROR-responsive elements (ROREs) on the FOXP3 promoter. Knockdown of RORC2 promotes high FOXP3 levels and decreased expression of pro-inflammatory cytokines IL-1β, IL-6, IL-17A IFN-γ and TNF-α in differentiating naïve T cells, suggesting that the role of RORC2 in Th17 cell development involves not only induction of Th17-characteristic genes, but also suppression of Treg-specific programs. Together, this study identifies RORC2 as a polarizing element in transcriptional cross-regulation and provides novel viewpoints on the control of immune tolerance versus effector immune responses.

Introduction

$CD4^+$ effector cells can be divided into different subsets according to their phenotype and function. Each subset is crucial for mounting an appropriate immune response to a certain type of pathogen (205, 224). Differentiation of naïve T helper cells towards a specific effector subset is therefore an important process in immune system homeostasis. T cell development is governed by lineage-specific transcription factors that regulate expression of characteristic surface receptors and cytokines. The Th1-specific transcription factor T-bet, for example, coordinates production of IFN-γ and TNF-α (16), while the GATA-binding protein 3 (GATA-3) regulates Th2 development including production of their characteristic cytokines IL-4, IL-5, IL-9 and IL-13 (20). Development of Th17 cells and expression of their specific cytokines IL-17A, IL-17F and IL-26 is orchestrated by the retinoic acid receptor-related orphan receptor γt/C2 (RORγt/RORC2) (153). While these effector subsets - Th1, Th2 and Th17 - are specialized to mount an immune response in order to tackle invading pathogens, regulatory T (Treg) cells in contrast control the strength and duration of the response and establish tolerance to harmless self- and non-self antigens. Failure in either development or function of Treg cells is a major cause of autoimmune and inflammatory disorders. Forkhead box protein 3 (FOXP3) is the master regulator of Treg cells. Retroviral gene transfer of FOXP3 is sufficient to convert naïve T cells into a regulatory T cell phenotype (67) and confers suppressor function on peripheral $CD4^+CD25^-$ T cells (68, 69). Expression of FOXP3 is regulated through interaction of several TCR-induced transcription factors, including NFAT, AP-1, cAMP response element-binding protein (CREB)/activating transcription factor (ATF), with the promoter or an enhancer element of the *foxp3* gene (74, 75, 80, 225, 226). However, isolated TCR-stimulation appears not to be sufficient for high levels of FOXP3 expression and IL-2, TGF-β and IL-27 have been reported to act as enhancing factors in FOXP3 gene regulation (76-78, 80, 82).

Cross-inhibition of T cell subsets during differentiation is an important mechanism to ensure expansion of the desired population. This is partially mediated through cytokines produced by one subset that inhibit development of other subsets. However, the lineage-specific transcription factors also play an important role in cross-regulation of T cell subpopulations. T-bet inhibits development of Th2 by binding to their master regulator GATA-3, preventing it from DNA binding and activation of Th2-specific genes (10, 11). Likewise, GATA-3 can

down-regulate STAT4, a transcription factor important for Th1 development (12). By binding to GATA-binding sites in the FOXP3 promoter, GATA-3 also negatively regulates FOXP3 expression and thereby inhibits Treg development (13). The Th17-driving transcription factor RORC2 is expressed by both Treg and Th17 cells, while only Treg cells express FOXP3 (14, 227, 228). In Treg cells, FOXP3 suppresses transcriptional activity of RORC2 by direct interaction, thereby inhibiting Th17 development (14, 15).

RORC2 belongs to the family of retinoic acid receptor (RAR)-related orphan receptors (RORs). This subfamily consists of three members: RORA (NR1F1, RORα, RZRα), RORB (NF1F2, RORβ, RZRβ) and RORC (NF1F3, RORγ, RZRγ). ROR genes generate several isoforms that differ only in their N-termini (168-170). Proteins of the ROR family display a typical nuclear receptor domain structure consisting of four domains: an N-terminal domain, a highly conserved DNA-binding domain (DBD), a hinge domain and a C-terminal ligand-binding domain (LBD). The DBD binds to DNA containing a so-called ROR-response element (RORE), which consists of the DNA sequence GGTCA as a core motive, preceded by an A/T-rich sequence (171, 172). In contrast to other nuclear receptors, ROR family proteins bind DNA as monomers (169, 171, 173). They can interact with both coactivators and corepressors to positively or negatively regulate transcription of target genes and are involved in a wide range of developmental processes. RORC plays a key role in the development of lymph nodes and Peyer's patches and in the regulation of thymopoiesis (170, 198-200).

In contrast to T-bet, GATA-3 and FOXP3, RORC2 has not been described to participate in cross-regulation of T cell subsets. Based on our observation that GATA3 inhibits FOXP3 expression (13), we hypothesized that the preference for Th17 over Treg cells might be mediated through restriction of the *foxp3* gene by RORC2. The present study describes a role for RORC2 in T cell cross-regulation by negatively regulating FOXP3 expression, which involves binding of RORC2 to the FOXP3 promoter region.

Materials and Methods

Isolation of naïve T cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors using Ficoll (Biochrom KG) density gradient centrifugation. $CD4^+$ T cells were purified using anti-CD4 magnetic beads and Detach-a-Bead antibodies (Dynal). $CD45RA^+$ cells were isolated using MACS magnetic beads (Miltenyi Biotec GmbH).

Differentiation of Treg and Th17 cells

$CD45RA^+$ T cells were stimulated with soluble anti-CD3 and anti-CD28 antibodies (4 µg/ml) in serum-free AIM-V medium (Life Technologies) supplemented with IL-2 (25 U/ml, Proreo Pharma AG). Anti-CD3 and anti-CD28 antibodies were produced using the hybridoma cell line OKT3 (American Type Tissue Collection) and the hybridoma cell line 15E8 (CLB). Treg cells were generated in the presence of TGF-β (5 ng/ml, R&D) and neutralizing anti-IL-12 (5 µg/ml, R&D) and anti-IFN-γ (1 µg/ml, R&D) antibodies. Th17 cells were differentiated as previously described (228) in the presence of TGF-β, IL-6 (20 ng/ml, PeproTech EC), IL-1β (10 ng/ml, PeproTech EC), IL-23 (10 ng/ml, eBioscience) and neutralizing anti-IL-12 and anti-IFN-γ antibodies. Dose titrations were performed and the optimal dose for each cytokine and antibody was determined in preliminary experiments.

Isolation of RNA, cDNA synthesis and quantitative RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed with TaqMan reverse transcription reagents (Fermentas) using random hexamer primers according to the manufacturer's protocol.

PCR primers were designed based on the sequences reported in GenBank using the Primer Express software version 1.2 (Applied Biosystems). Primers used for relative quantification are listed in table 1. All primers are spanning intron-exon borders and have been verified for efficacy over a 4-log concentration range. The prepared cDNAs were amplified using iTaq SYBR Green Supermix with ROX (BioRad) according to the manufacturer's recommendations in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Relative quantification and calculation of the range of confidence was performed using the

comparative ΔΔCT method. EF-1α was used as endogenous control. All amplifications were conducted in triplicates.

Determination of cytokine concentration

T cells were cultured for 5 d under Th17 conditions. Before restimulation with soluble anti-CD3 and anti-CD28 antibodies (4 μg/ml), the cells were washed to remove the cytokines used for differentiation. Supernatants were collected 48 h after restimulation and concentrations of IL-1β, IL-2, IL-5, IL-6, IL-13, IL-17A, IFN-γ and TNF-α were determined by cytometric bead array (BioRad) according to the manufacturer's protocol.

Western blotting

Protein extraction was performed as follows: The cells were pelleted and resuspended in buffer C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, protease inhibitors (Roche Diagnostics), and 0.1% Nonidet P-40), and incubated at 4 °C with agitation for 15 min. Insoluble material was removed by centrifugation and the supernatants were diluted 1/3 with buffer D (as buffer C, but without NaCl). Total protein concentrations were determined by a colorimetric protein assay (BioRad). Samples were loaded next to a prestained protein-mass ladder (Invitrogen Life Technologies) on a NuPAGE 4–12% bis-Tris gel (Invitrogen Life Technologies). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Unspecific binding was blocked with TBS-Tween 5% BSA. After blocking, the membrane was incubated with an anti-RORγ antibody (Santa Cruz Biotechnology) in a 1:500 dilution in blocking buffer over night at 4 °C. Next, the membrane was washed and incubated with an anti-rabbit HRP labeled Ab (Cell Signaling, 1:3000) at RT for 1 h. The blot was developed using an ECL plus Western Blot Detection System (GE Healthcare) and visualized with a LAS 1000 camera (Fuji). To confirm equal sample loading and transfer, membranes were stripped, re-blocked and re-probed using an anti-GAPDH antibody (6C5, Ambion Ltd.).

Flow cytometry

Cells were stained with the CD25 mAb (Beckman Coulter) prior to FOXP3 intracellular staining, which was performed according to the manufacturer's protocol using

AlexaFluor®488 anti-human FOXP3 Flow kit (295D; Biolegend). Matched isotype controls were used at the same protein concentration as the respective antibodies.
Cell acquisition by flow cytometry was done on a Four-color FACS EPICS™ XL-MCL (Beckman Coulter) using the software Expo™32 version for data acquisition and evaluation.

Amplification of FOXP3 promoter fragments

FOXP3 promoter fragments were amplified by conventional PCR using a biotinylated reverse primer (5'-bio-ACCTTACCTGGCTGGAATCACG-3') situated 177 bp downstream of the TSS. Multiple forward primers were designed to generate FOXP3 promoter fragments of decreasing length (table 2). The position of the primers are depicted in Fig. 19*A*. Reactions were conducted in 75 mM Tris HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 6 µg/ml template DNA (pGL4 FOXP3 -1210 /+177) and 1.25 U of Taq DNA polymerase (Fermentas). The same PCR conditions were used for the amplification of all the products: Initial denaturation step (2 min, 94 °C), 42 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, final elongation step (7 min, 72 °C). PCR products were purified by ethanol precipitation. For this purpose 1/10 volume of 3 M sodium acetate and 3 volumes of 100% ethanol were added to the PCR products and samples were mixed, precipitated for 1 h at -80 °C and harvested by centrifugation (30 min, maximum speed, 4 °C). The supernatant was removed, residual ethanol was allowed to evaporate and the pellet was eluted in blocking buffer (PBS, 0.1% BSA, 0.05% Tween20). To quantify the FOXP3 promoter fragments, samples were loaded next to a MassRuler DNA Ladder Mix (Fermentas) on a 1% agarose gel and quantified using the AIDA image analyzer software (Raytest). For fragment F11 and for competitor oligonucleotides, sense and antisense oligonucleotides (Microsynth, sequences in table 2) were annealed for 10 min at 95 °C.

FOXP3 promoter ELISA

Protein extraction was performed as described for Western blotting. 384-well flat bottom plates were precoated with streptavidin (Pierce) and were washed 3 times with washing buffer (PBS, 0.05% Tween20). Biotinylated FOXP3 promoter fragments or consensus sequences were added (1 pmol per well; 50 fmol/µl) and incubated for 1 h at RT. Upon 3 wash cycles, nuclear extracts were added at concentration of 1 µg/µl and incubated overnight at 4 °C in the presence of 10 µg of poly(deoxyinosinic-deoxycytidylic) acid (Sigma-Aldrich) to block

unspecific binding. The plate was washed 3 times with buffer C/D and then incubated with the primary antibody (rabbit antiRORC, 1:200 in buffer C/D, Biolegend) at 4 °C for 2 h. After 3 wash cycles with buffer C/D, a secondary antibody (anti rabbit IgG-HRP, 1:3000 in buffer C/D, Cell Signaling) was added and the plate was incubated for 1 h at 4 °C. The wells were washed 4 times with buffer C/D before substrate reagent was added (R&D system). The colorimetric reaction was stopped after 2 to 5 min by adding 2 M H_2SO_4. Absorbance at 450 nm was measured using a Mithras microplate reader (Berthold Technologies).

Transfections

Naïve T cells were pre-activated overnight in serum-free AIM-V medium (Life Technologies) containing soluble anti-CD3 and anti-CD28 antibodies (4 µg/ml). 6-10 x 10^6 T cells were transfected with 3 µg of pcDNA3.1-empty, pcDNA3.1-RORC2FL or pcDNA3.1-RORC2ΔDBD plasmids in 100 µl of Nucleofector™ solution (Amaxa Biosystems) using the T-23 program. SiRNA oligos (Ambion) were transfected at a concentration of 30 nM. Optimal concentrations were determined in preliminary experiments (Fig. 16). Two different siRNA oligos were tested and oligo siRNA1 was used in subsequent experiments. SiRNA sequences are listed in table 3. 8 h after transfection with plasmids or siRNA, the medium was replaced with fresh, serum-free AIM-V medium and cells were stimulated with anti-CD3 and anti-CD28 antibodies (4 µg/ml) or as indicated.

HEK cells were plated in 6 well dishes and transfected with of pcDNA3.1-empty, pcDNA3.1-RORC2FL or pcDNA3.1-RORC2ΔDBD plasmids at 90-95% confluence using the lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction.

Reporter gene assays

6-10 x10^6 naïve T cells were co-transfected with of 3 µg of the pGL4-empty or the pGL4-FOXP3 promoter luciferase reporter vector and 2 µg of pcDNA3.1-empty, pcDNA3.1-RORC2FL or pcDNA3.1-RORC2ΔDBD plasmids in 100 µl of Nucleofector™ solution. Electroporation was performed using the T-23 program. After 8 h, the medium was replaced with fresh serum-free AIM-V medium and cells were stimulated with anti-CD3 and anti-CD28 antibodies (4 µg/mL) for another 12 h until luciferase activity in cell lysates was measured using the dual luciferase assay system (Promega Biotech) in a Berthold Lumat LB 9507 luminometer according to the manufacturer's instructions.

Plasmids

The human FOXP3 promoter was cloned into the pGL4 vector (Promega Biotech) to generate the pGL4 FOXP3 -1210/+177 as previously described (74). Human RORC2FL and RORC2ΔDBD were cloned into a pcDNA3.1 vector (Invitrogen) using the primers listed in table 4.

Bioinformatics

Alignments of the human FOXP3 promoter were performed using the Genomatix software (http://www.genomatix.de).

Table 1. Primers used for RT-PCR

EF-1α fw	CTGAACCATCCAGGCCAAAT
EF-1α rv	GCCGTGTGGCAATCCAAT
FOXP3 fw	GAAACAGCACATTCCCAGAGTTC
FOXP3 rv	ATGGCCCAGCGGATGAG
RORC2 fw	CAGTCATGAGAACACAAATTGAAGTG
RORC2 rv	CAGGTGATAACCCCGTAGTGGAT

Table 2. Primers and oligonucleotides used for promoter ELISA

P-1116fw	GACATGTTCAGCACTGTATCTGACCC
P-1006fw	AGTAAAAGACCCCAAAGGCTGAGG
P-896fw	CATTAATATTGGGGACCTGCTAGGG
P-511fw	TTCCCATCCACACATAGAGC
P+1fw	AAGCCAGGCTGATCCTTTTCTGT
P+176rv-bio	bio-ACCTTACCTGGCTGGAATCACG
F11 sense	TCTGGTACAGTGGGATGTACCCAGCTACCGTGATTCCA GCCAGGTAAGGT
F11 antisense	ACCTTACCTGGCTGGAATCACGGTAGCTGGGTACATCC CACTGTACCAGA
Competitor WT sense	TACGCAGCTGAAATGAGGTCAGGCAGACTA
Competitor WT antisense	TAGTCTGCCTGACCTCATTTCAGCTGCGTA
Competitor MUT antisense	TACGCAGCTGAAATGCCTAGCGGCAGACTA
Competitor MUT antisense	TAGTCTGCCGCTAGGCATTTCAGCTGCGTA

Table 3. SiRNA oligos

SiRNA1 sense	CAAUCUCUCUUAUCCUUGAtt
SiRNA1 antisense	UCAAGGAUAAGAGAGAUUGtg
SiRNA2 sense	CCCUGACAGAGAUAGAGCAtt
SiRNA2 antisense	UGCUCUAUCUCUGUCAGGGag

Table 4. Primers used for cloning

RORC2FLfw	GAGAGGTACCGACATGAGAACACAAATTGAAGTGATCCC
RORC2ΔDBDfw	GAGAGGTACCGACATGCTGTCAAGTTCGGCCGCAT
RORC2rv	ATATGCGGCCGCTCACTTGGACAGCCCCACAGGT

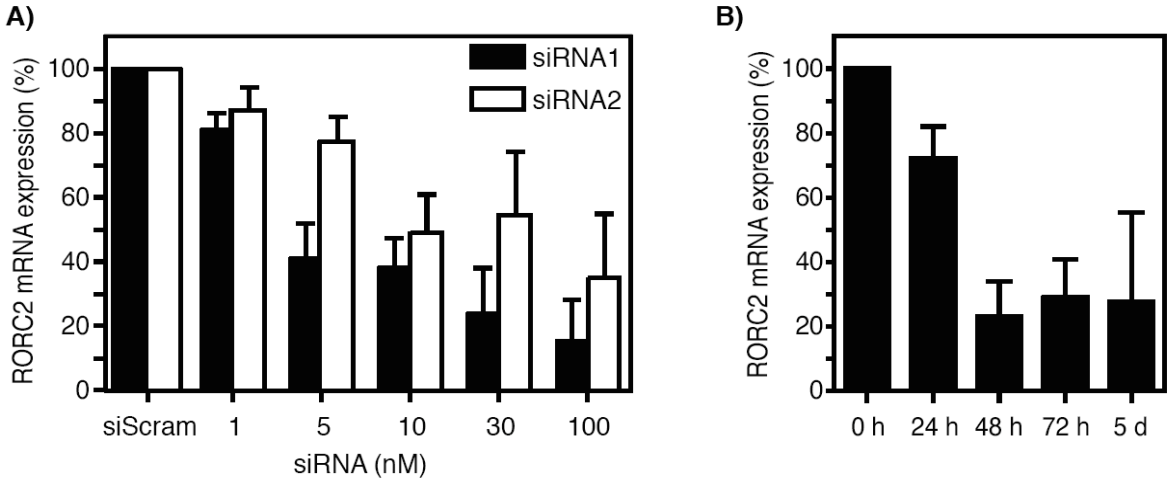

Fig 16. Dose-titration and time-kinetic of siRNA oligonucleotides against RORC2. ***A***, Naïve $CD4^+$ T cells were transfected with two different siRNA oligonucleotides against RORC2 at increasing concentrations. After 48 h RORC2 mRNA expression was determined by RT-PCR. Levels in scrambled siRNA transfected cells was set to 100%. Bars represent mean and standard deviation of 3 independent experiments. ***B***, Naïve $CD4^+$ T cells were transfected with siRNA1 at a concentration of 30 nM. RORC2 mRNA levels were determined by RT-PCR after different time points. Expression at time-point 0 h was set to 100% and levels at each time points were normalized to levels in scrambled siRNA transfected cells at the same time point. Bars represent mean and standard deviation of 3 independent experiments. (siScram = scrambled siRNA)

Results

Over-expression of RORC2 reduces FOXP3 expression

While RORC2 activity is inhibited by FOXP3 in Treg cells, this inhibitory function appears to be abolished in Th17 cells. Considering the key roles of lineage-specific transcription factors in mutual inhibition of T cell subsets, RORC2 itself represents a candidate for this unidentified suppressor of FOXP3. In order to test whether RORC2 has the capacity to suppress FOXP3 expression, RORC2 was cloned as a fulllength construct (RORC2FL) as well as a truncated version lacking the DNA binding domain (RORC2ΔDBD), which was used as negative control. Naïve $CD4^+$ T cells were transfected with these plasmids and Western blot analysis was performed to confirm recombinant expression of the proteins (Fig. 17*A*). The molecular weight of the proteins observed on the gel were in accordance with the calculated molecular weight of app. 56 kDa for RORC2FL and 48 kDa for RORC2ΔDBD. The additional faint band at app. 50 kDa was due to unspecific binding of the antibody. To test the influence of RORC2 on FOXP3 expression, transfected T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 and TGF-β and FOXP3 levels were determined by RT-PCR (Fig. 17*B*). In cells transfected with RORC2FL, FOXP3 mRNA levels decreased about 60 percent and (66±23% after 48 h and 61±14% after 5 d). For control, in cells transfected with the DNA-binding deficient RORC2ΔDBD mutant, FOXP3 levels were comparable to mock transfected cells. To investigate whether RORC2 can change FOXP3 protein levels, cells transfected with the RORC2 constructs were analyzed by flow cytometry after 5 d (Fig. 17*C*). Consistent with the results obtained by RT-PCR, RORC2FL-transfected cells showed a markedly decreased FOXP3 staining (14.0±3.2%) compared to mock (22.0±1.9%) or RORC2ΔDBD transfected cells (23.2±1.8%). Taken together, these results demonstrate an inhibitory role for RORC2 on FOXP3 mRNA and protein expression by a DNA binding-dependent mechanism.

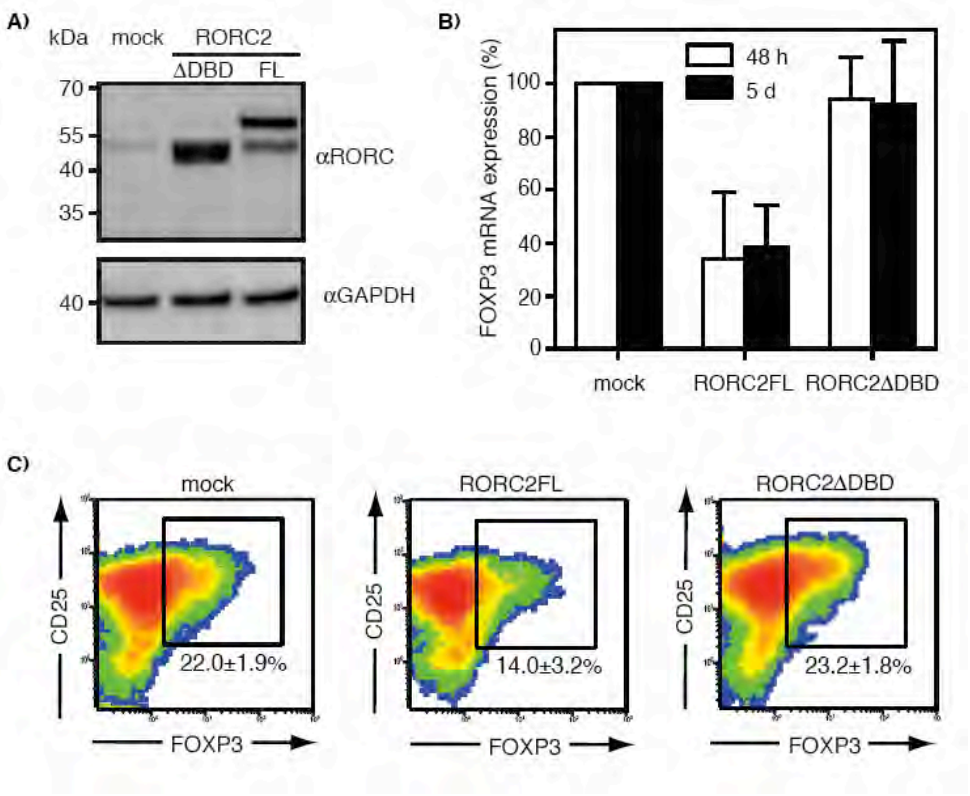

Fig 17. Over-expression of RORC2 inhibits FOXP3 expression. ***A***, Naïve $CD4^+$ T cells were transfected with RORC2ΔDBD and RORC2FL and expression of the proteins was analyzed by Western blot. ***B*** and ***C***, Naïve $CD4^+$ T cells were transfected with RORC2FL and RORC2ΔDBD. 8 h after transfection the cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 and TGF-β. ***B***, FOXP3 mRNA levels were determined by RT-PCR after 48 h and 5 d and levels in untreated cells were set to 100%. *C*, FOXP3 protein expression was measured by FACS after 5 days. Bars/numbers indicate mean and standard deviation of at least 3 independent experiments.

Knockdown of RORC2 increases FOXP3 levels

The observation that high RORC2 levels result in a down-regulation of FOXP3 led us to the hypothesis that a decrease in RORC2 might enhance the expression of FOXP3. To test this, RORC2 was knocked-down using siRNA oligonucleotides. Two different oligonucleotides were tested and a dose-titration as well as a time-kinetic was performed to validate RORC2 knockdown (Fig. 16). Cells were transfected with siRNA against RORC2 or with a scrambled siRNA as negative control and then cultured with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 and TGF-β. After 48 h, mRNA levels of RORC2 and FOXP3 were determined by RT-PCR (Fig. 18*A*). In cells transfected with siRNA against RORC2, levels of RORC2 were knocked-down by 76.1±14% compared to untransfected cells, while no effect was observed with scrambled siRNA, confirming the specificity of the assay. In cells with

reduced RORC2 levels due to siRNA treatment, a 3-fold increase in FOXP3 was observed. To analyze FOXP3 expression on protein level, cells were cultured for 5 d under the same conditions and then subjected to FACS analysis (Fig. 18*B*). Consistent with the results obtained by RT-PCR, numbers of FOXP3 positive cells increased in the presence of siRNA against RORC2 compared to scrambled siRNA-transfected cells (from 20.1±2.0% to 28.5±4.0%). These data reveal that down-regulation of RORC2 increases FOXP3 expression and they are in agreement with the results above showing reduced FOXP3 expression after RORC2 over-expression.

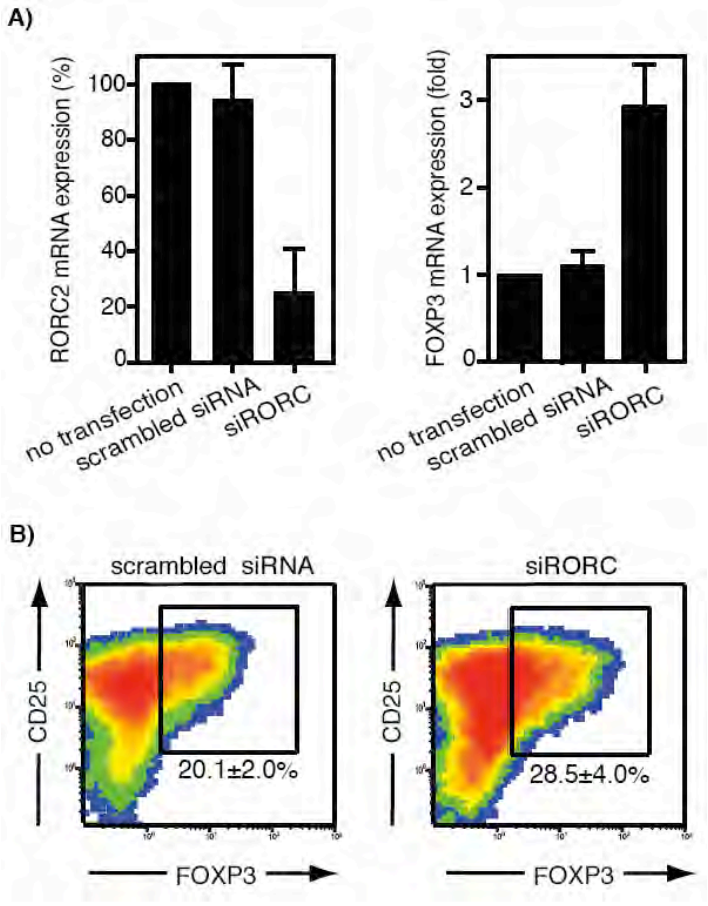

Fig 18. SiRNA-mediated knockdown of RORC2 increases FOXP3 expression. Naïve $CD4^+$ T cells were transfected with siRNA oligonucleotides targeting RORC2. 8 h after transfection the cells were stimulated with anti-CD3, anti-CD28 antibodies in the presence of IL-2 and TGF-β. mRNA levels of RORC2 (left) and FOXP3 (right) were determined by RT-PCR after 48 h and 5 d **(*A*)**. Levels in untreated cells were set to 100% or to 1, respectively. Bars represent mean and standard deviation of at least 3 independent experiments. FOXP3 protein expression was measured by FACS after 5 d **(*B*)**. Numbers indicate mean and standard deviation of at least 3 independent experiments.

RORC2 binds to the FOXP3 promoter at RORE -942 and RORE +115

Transcription factors of the nuclear receptor family like RORC2 control gene expression by binding to the promoter of their target genes. To investigate the mechanism by which RORC2 controls FOXP3 expression, we first analyzed the FOXP3 promoter sequence for the presence of ROREs, putative binding sites for RORC2 (Fig. 19*A*). The sequence from bp -1242 until bp +223 contains four ROREs, three upstream (at -1096, -942 and -847) and one downstream the transcription start site (at +115). These putative binding sites for ROR-family members are conserved among the FOXP3 promoters of different species (Fig. 19*B*). Direct binding of RORC2 to the FOXP3 promoter was assessed using a FOXP3 promoter ELISA system (Fig. 20*A*). In this assay, either consensus sequences of 30 bp containing one RORE or promoter fragments spanning bp -1116 to +177 were coated on ELISA plates. Plates were incubated with extracts of HEK cells transfected with RORC2 or an empty vector as a control and binding of RORC2 was quantified by chemiluminescence following incubation with the appropriate antibodies. Binding of RORC2 to the wildtype, but not to the mutated consensus sequence was observed, validating the specificity of the assay. Absorbance was even slightly higher when extracts of HEK cells transfected with RORC2 were incubated with the FOXP3 promoter sequence, while incubation with extracts from empty-vector-transfected cells produced only background levels of absorbance. These results suggest that RORC2 binds to one or more of the RORE on the FOXP3 promoter. The sequence specificity of the binding was further confirmed by addition of soluble oligonucleotides, which abolished binding by competition when they contained the wild type, but not a mutated consensus sequence.

To investigate which of the four ROREs are important for binding of RORC2 to the FOXP3 promoter, serial deletions of the FOXP3 promoter were constructed and fragments were coated on an ELISA plate (Fig. 20*B*). Binding of RORC2 to the promoter fragment lacking RORE-1096 was equal to binding observed using the fragment containing all four ROREs. However, when RORC2 was incubated with a construct lacking RORE-942, a drop in absorbance was detected, suggesting that RORE-942 is relevant for binding of RORC2 to the promoter. Deletion of RORE-847 did not substantially alter the absorbance of the promoter ELISA, while a clear drop in absorbance was found in the fragment lacking also RORE+115. RORC2 has been described to compete with NFAT for DNA binding (229). As three putative NFAT binding sites are located on the FOXP3 promoter (Fig. 19*A*), a construct lacking these

sites was analyzed for RORC2 binding (Fig. 20*B*). However, no difference in binding was observed.

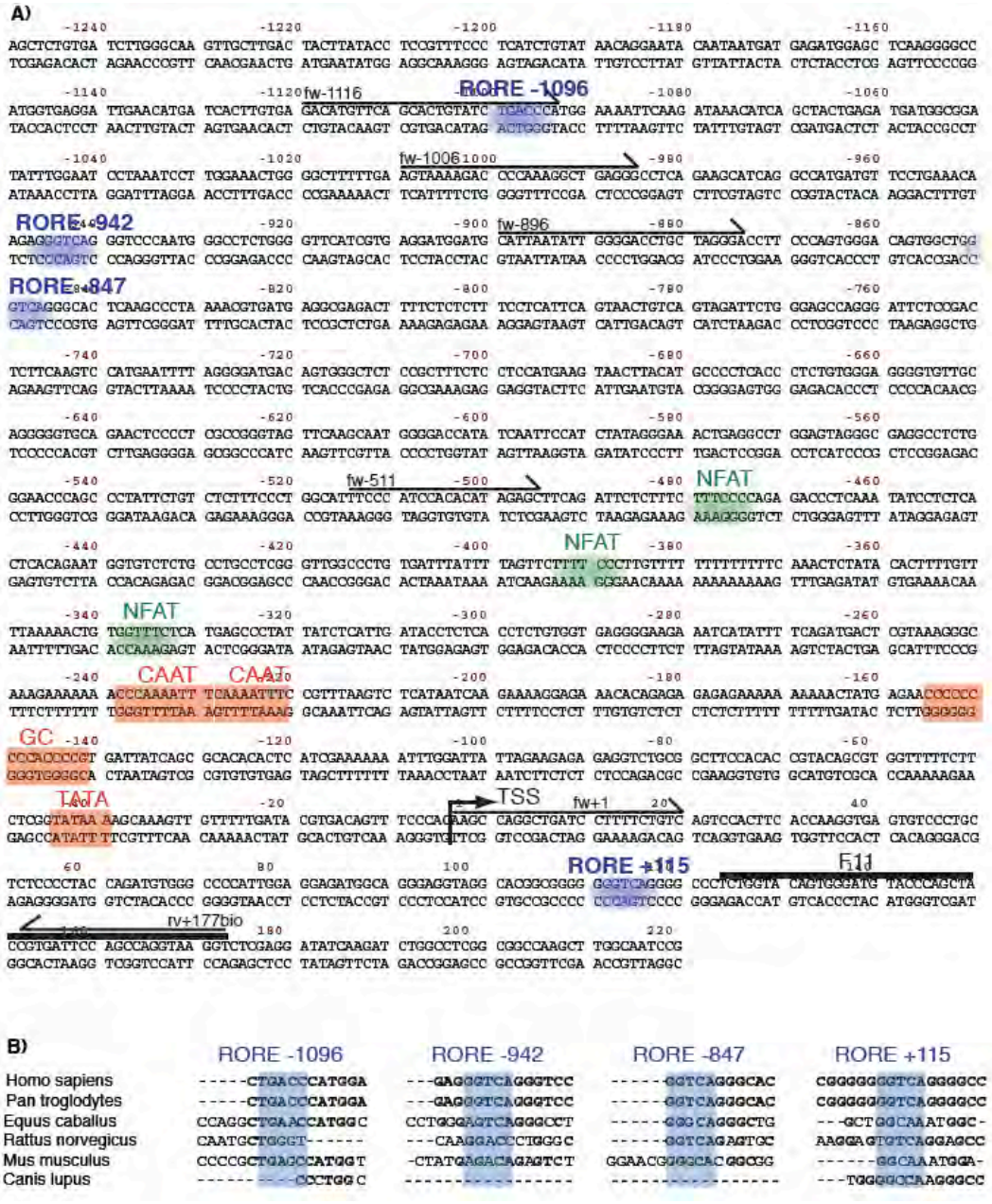

Fig 19. The FOXP3 promoter contains conserved ROREs. ***A,*** Sequence of the FOXP3 promoter from bp -1246 to bp +223. ROREs are marked in blue, NFAT binding sites are marked in green. CAAT, GC and TATA boxes are indicated with orange boxes. Primers used for generation of the promoter fragments are marked with black arrows, TSS = transcription start site. ***B,*** FOXP3 promoters of different species were aligned using the genomatix software. Conserved bases are highlighted in bold, RORE core sequences are marked with blue boxes.

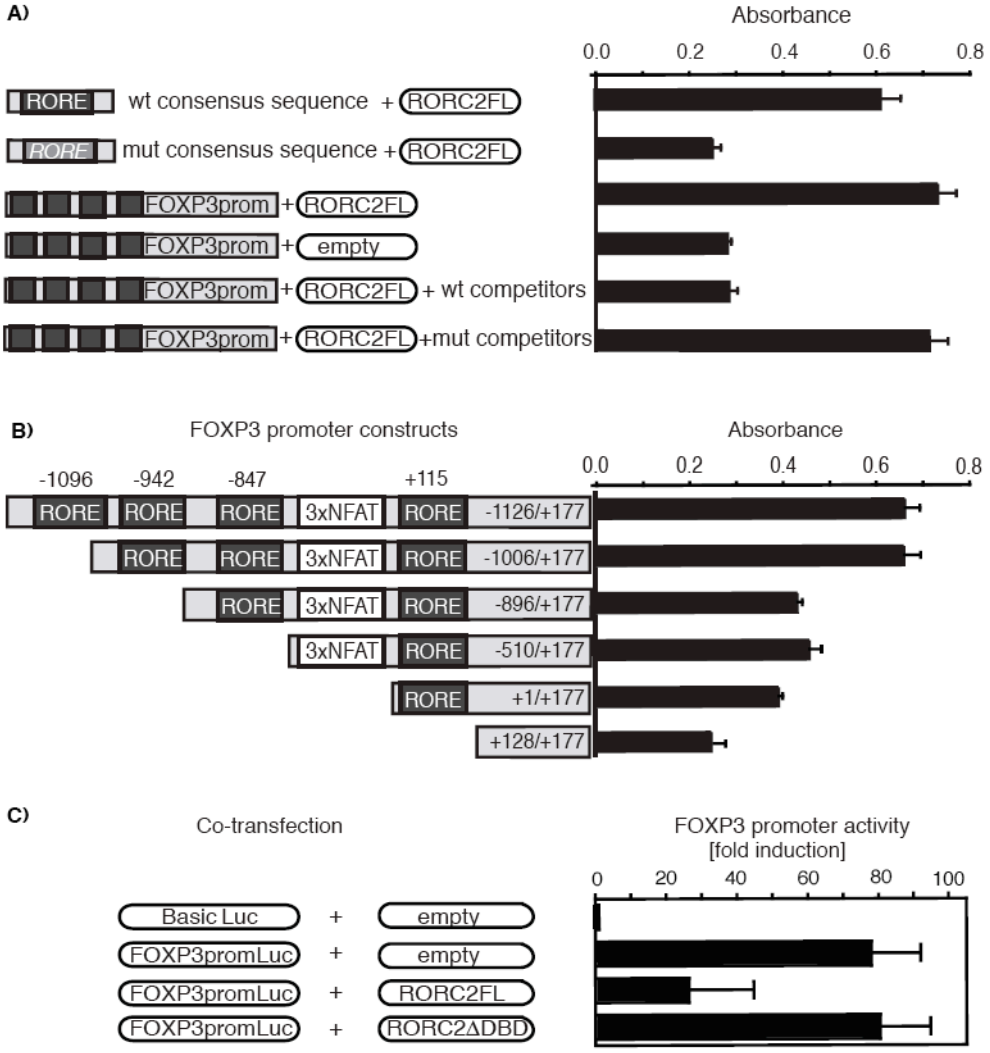

Fig 20. RORC2 binds to the FOXP3 promoter. ***A,*** Nuclear extracts of HEK cells transfected with either RORC2 or an empty vector were incubated with a wildtype or a mutated RORE consensus sequence or with the FOXP3 promoter in the presence or absense of competitor oligonucleotides. Binding of RORC2 was quantified using the FOXP3 promoter ELISA assay. Bars show mean and standard deviations of triplicates representative of three independent experiments. ***B,*** Nuclear extracts of HEK cells transfected with RORC2 were incubated with FOXP3 promoter fragments of different length. Binding of RORC2 was quantified using the FOXP3 promoter ELISA assay. Bars show mean and standard deviations of triplicates representative of three independent experiments. ***C,*** Naïve $CD4^+$ T cells were co-transfected as indicated. 8 h after transfection the cells were stimulated with anti-CD3, anti-CD28 antibodies for 12 h until luciferase activity was measured. Bars represent mean and standard deviation of 3 independent experiments. (wt = wildtype, mut = mutated)

To find out whether binding to the FOXP3 promoter is required for RORC2-mediated inhibition, a FOXP3 promoter luciferase assay was performed (Fig 20*C*). Co-transfection of RORC2FL and a luciferase plasmid containing the FOXP3 promoter resulted in a 67% reduced luciferase activity compared to the luciferase activity in cells transfected with the luciferase vector and an empty vector (26.4±17 fold versus 79.3±13 fold increase to the basic luciferase plasmid). In contrast, co-transfection with RORC2ΔDBD generated a luciferase activity comparable to empty vector co-transfection. Taken together, these results suggest that RORC2 inhibits FOXP3 expression by binding to its promoter at RORE-942 and RORE+115.

Knockdown of RORC2 shifts Th17 cells towards T cells with a less pro-inflammatory but more regulatory phenotype

To investigate whether these findings have an implication in T cell differentiation - especially in the balance between Treg and Th17 cells - we analyzed whether lack of RORC2 shifts Th17 cells towards a Treg phenotype. Therefore, RORC2 was down regulated in naïve T cells using siRNA and the cells were cultured under either Treg or Th17 differentiation conditions. As expected, in control settings (i.e. scrambled siRNA-transfected cells) FOXP3 mRNA levels were clearly higher in Treg than in Th17 (Fig 21*A*). However, in Th17 cells with down-regulated RORC2 levels, FOXP3 expression was significantly increased, almost reaching levels in siRNA treated Treg cells. Similarly, frequencies of FOXP3 positive cells in siRNA transfected Th17 cells were comparable to frequencies in cells cultured under Treg conditions, while in cells transfected with control siRNA a considerably smaller fraction of Th17 cells was FOXP3 positive (Fig 21*B*). We further analyzed the cytokine profile of Th17 cells carrying reduced RORC2 levels during differentiation due to siRNA treatment (Fig 21*C*). As expected and previously reported (162, 163), IL-17A levels were markedly reduced in these cells. Moreover, these cells also showed a decreased expression of other pro-inflammatory cytokines like IL-1β, IL-6, IFN-γ and TNF-α (Fig 21*C* left), while no changes in IL-5 and IL-13 levels were observed (Fig 21*C* right), suggesting a specific effect. These results indicate that Th17 cell development is impaired in RORC2-depleted cells and that these cells instead differentiate towards a less pro-inflammatory but more tolerating subset.

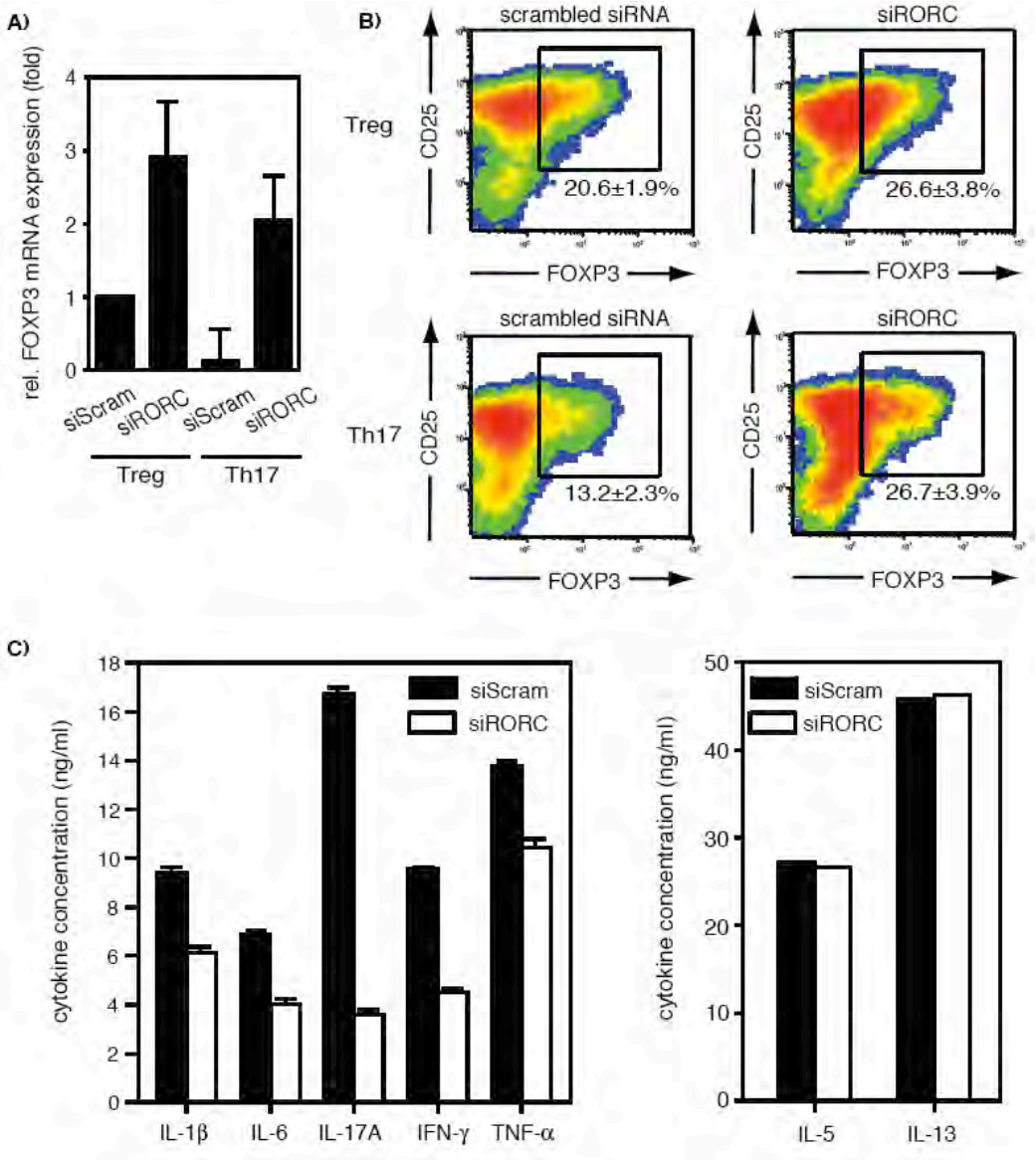

Fig 21. Down-regulation of RORC2 shifts Th17 cells towards less inflammatory phenotype. Naïve $CD4^+$ T cells were transfected with siRNA against RORC2. 8 h after transfection the cells were cultured under Treg or Th17 conditions for 5 d. ***A,*** After 6 h of restimulation with anti-CD3, anti-CD28 antibodies, FOXP3 mRNA levels were measured by RT-PCR. Levels in scrambled siRNA transfected Treg cells were set to 1. Bars represent mean and standard deviation of 3 independent experiments. ***B,*** After 6 h of restimulation with PMA and Ionomycin, FOXP3 expression was analyzed by FACS. Numbers indicate mean and standard deviation of 3 independent experiments. ***C,*** After 48 h of restimulation with anti-CD3 and anti-CD28 antibodies cytokine concentration was analyzed by Cytometric bead array. Bars represent standard errors of duplicates. Data shown are representative of 5 independent experiments. (siScram = scrambled siRNA)

Discussion

The current study demonstrates repression of FOXP3 by the Th17 cell-promoting transcription factor RORC2 and thus identifies a novel role for RORC2 in T cell polarization. Over-expression of RORC2 decreased the levels of FOXP3 on both mRNA and protein level. This relationship was confirmed by siRNA-mediated knockdown of RORC2, which increased FOXP3 expression, indicating an inverse correlation of the two factors. Binding to the promoter of FOXP3 is necessary for RORC2-mediated repression, as overexpression of RORC2 lacking the DNA-binding domain did not inhibit FOXP3 expression. Moreover, no decrease in luciferase activity was observed upon co-transfection with the DBD-deficient construct. To further investigate the mechanism of repression, we analyzed the FOXP3 promoter sequence from bp -1242 until bp +223 and identified four putative RORC2-binding sites. In agreement with the fact that ROR transcription factors bind DNA as monomers (169, 171, 173), all ROREs identified consist of a single independent motif. Three of these ROREs are located in a poorly characterized part of the FOXP3 gene, slightly upstream of the core promoter and the region, which we previously identified as an inductive area for TCR-inducible signals such as NFAT and AP-1 (74, 230)(74, 230). A fourth RORE is situated downstream the transcription start site. Using an ELISA-based system, we detected binding of RORC2 to RORE-942 and to a lesser extend to RORE+115. ROREs consist of the DNA sequence GGTCA as a core motive and are often preceded by an A/T-rich sequence (171, 172). This A/T-rich sequence of RORC2 binding sites is also found upstream of RORE-942. Altogether, these experiments reveal that repression of FOXP3 by RORC2 involves physical binding of RORC2 to two ROREs on its promoter, a mechanism commonly observed for ROR-family members.

ROR-family transcription factors induce repression of target genes by recruiting co-repressors, which induce chromatin de-acetylation and therefore gene repression (183-185). Several co-repressors have been described to interact with ROR family members and it remains to be determined, which of them mediates the repression of the *foxp3* gene observed in this study. Retinoic acid has been reported to be a key factor in regulating the balance of Treg versus Th17 cells (86-88, 231). Although retinoic acid seems to mediate these effects by its cognate receptor retinoic acid receptor (RAR) α (89, 232), the mechanism possibly also involves RORC2, as retinoic acid has been found to bind to RORC2 and to inhibit its

transcriptional activity (178). Therefore, retinoic acid might favor Treg cell development by binding and inhibiting RORC2, leading to an increased FOXP3 expression. However, addition of retinoic acid did not change extract binding properties to the ROREs on the FOXP3 promoter (data not shown).

Beside co-repressor-mediated suppression, RORC2 might also inhibit FOXP3 expression by competition with NFAT, which is important for TCR-induced *foxp3* gene activation (74), as RORC2 has been shown to bind to NFAT binding sites and to inhibit its transcriptional activity (229). In context of the *foxp3* gene, we did not find binding of RORC2 to NFAT binding sites, suggesting an alternate mechanism of repression. Since IL-2 plays a major role in FOXP3 induction (76, 77) and RORC2 has been shown to suppress IL-2 production (170), it may exert its inhibitory function through negative regulation of autocrine IL-2 secretion. In fact, levels of IL-2 dropped significantly upon siRNA-mediated RORC2 knockdown (data not shown).

The siRNA-induced RORC2-knockdown during differentiation of Th17 cells led to a phenotype characterized not only by high FOXP3 expression, but also by low levels of pro-inflammatory Th17 and Th1 cytokines such as IL-1β, IL-6 and IL-17A as well as IFN-γ and TNF-α, respectively. This suggests an important role for RORC2 in the inhibition of Treg development under inflammatory conditions. Reduced levels of IL-17A are presumably a direct consequence of RORC2 knockdown, which is known to regulate IL-17A expression on a transcriptional level (167). In contrast, the decrease in IFN-γ is a novel observation and might be caused indirectly by the increased FOXP3 expression, as FOXP3 has been shown to repress IFN-γ production (233).

Crossover inhibition of T cell subsets during differentiation is an important mechanism to ensure polarization and clonal expansion of the most efficient subset. The presented data highlight a new role for the Th17 cell master regulator RORC2 in the inhibition of the Treg-specific transcription factor FOXP3, thereby revealing a new mechanism of Treg-Th17 cell cross-regulation. Both T cell subsets require TGF-β for their development, whereas Th17 cells additionally need inflammatory cytokines (150-152, 162, 228). While the Th17 cell-inducing cytokines IL-6, IL-21 and IL-23 favor RORC2 over FOXP3 expression (90-93, 227, 234), IL-2, retinoic acid, as well as IL-27, which inhibits Th17 cells and enhances TGF-β-induced FOXP3 expression (82, 235-237), might shift the balance towards FOXP3 expression and Treg cell development. Based on these findings and the results of our study, we propose a

model in which FOXP3-mediated inhibition of RORC2 dominates under non-inflammatory conditions, while inflammation favors RORC2 and suppression of FOXP3 by RORC2 (Fig. 22).

The current report demonstrates that RORC2 is involved in the polarization of T cells by negative regulation of FOXP3 and therefore reveals an important pathway for future developments of drugs acting on the delicate balance between immunity and tolerance.

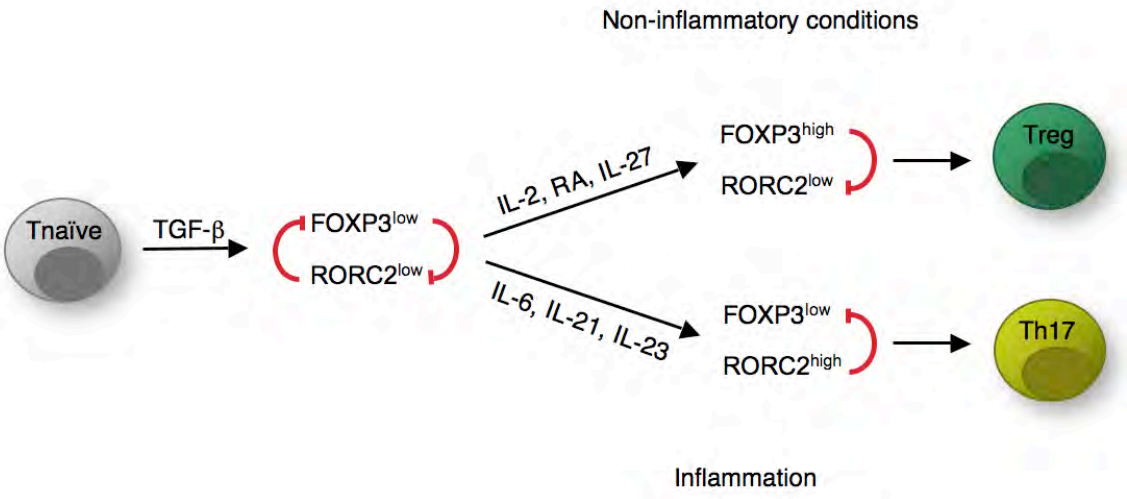

Fig 22. Mutual regulation of the transcription factors FOXP3 and RORC2. TGF-β induces both FOXP3 and RORC2. Under non-inflammatory conditions, mediators like IL-2, retinoic acid (RA) or IL-27 enhance TGF-β-induced FOXP3 expression. FOXP3 inhibits DNA-binding of RORC2 and thereby its transcriptional activity through physical interaction. As FOXP3 levels predominate under these conditions, no active RORC2 is available to repress the *foxp3 gene* and to activate Th17 cell programs. FOXP3 levels further increase, promoting the development of Treg cells. During inflammation, Th17-differentiating cytokines such as IL-6, IL-21 and IL-23 enhance RORC2 expression and/or inhibit FOXP3 expression, thereby favoring RORC2 over FOXP3. RORC2 further inhibits FOXP3 expression and induces transcriptional programs leading to Th17 cell development.

2.3 Statement of contribution to publications

I have performed all the experiments for both publications except for the suppression assay, which was performed by Nadia Ouaked. For the publication entitled “RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter” I used the FOXP3 promoter in the pGL4 vector that ~~has been~~ previously cloned by Pierre-Yves Mantel.

3 DISCUSSION

The differentiation of naïve T cells towards Th17 or Treg cells must be tightly controlled in order to establish equilibrium between protective immune responses and tolerance against harmless (self-) antigens. The current thesis demonstrates that when stimulated by TGF-β, IL-1β, IL-6 and IL-23, naïve human T cell differentiate into Th17 cells, a population that potently induces pro-inflammatory mediators in bronchial epithelial cells. Over-expression and knockdown experiments revealed that RORC2 plays a decisive role in T cell differentiation by negative regulation of the *foxp3* gene, thereby favoring Th17 over Treg cell development.

3.1 Requirements for Th17 cell differentiation

While there is consent about the mediators inducing differentiation of murine Th17 cells, the factors required for human Th17 development are controversially discussed. The present thesis demonstrates that TGF-β, the pro-inflammatory cytokines IL-1β and IL-6, as well as IL-23 are required for the development of a functional Th17 effector population, characterized by high production of IL-17A and IL-17F.

While other T cell subsets depend on IL-2 for their survival, Th17 cells seem to be rather inhibited by IL-2, at least in mice (157). In agreement with these observations, Th17 cell development was not further enhanced by IL-2, but we did not observe an inhibition of Th17 cells development, neither. IL-21, a common gamma chain family member like IL-2, has been suggested to act as a survival factor for Th17 cells and may even substitute for IL-6 (92, 154, 158). Consistently, we noticed a slight IL-17A induction by TGF-β and IL-21. However, the addition of IL-21 to the cytokine cocktail TGF-β, IL-1β, IL-6 and IL-23 did not lead to a further enhancement of IL-17A and IL-17F. As IL-21 is induced by IL-6 and produced by Th17 cells themselves (92), it is likely that the cells cultured with TGF-β, IL-1β, IL-6 and/or

IL-23 secrete substantial levels of IL-21, which then acts in an autocrine manner, overcoming an effect of supplemental IL-21.

The necessity for TGF-β in the development of human Th17 cells is currently under debate. In serum-free conditions we found a crucial role for TGF-β in the induction of Th17-specific genes IL-17A, IL-17F and RORC2. The discrepancies between different studies might be due to differences in culture conditions such as usage of serum. It is assumed that an appropriate concentration of TGF-β is crucial for Th17 cell differentiation and that high doses of TGF-β rather suppress IL-17, as observed for other cytokines. Therefore, low levels of TGF-β present in serum might be sufficient for Th17 cell differentiation and the concentration might exceed the optimum upon additional TGF-β supplementation. Furthermore, TGF-β is possibly required at early stages of Th17 development, but at later stages it rather inhibits IL-17 production. This hypothesis is supported by our observation showing that neutralizing anti-TGF-β antibodies decrease IL-17 production in naïve T cells cultured under Th17 conditions, whereas these antibodies do not affect IL-17 production in memory cells. Consistent with these results, previous studies suggest that TGF-β is not required for IL-17 induction in memory cells and that an optimal induction of IL-17 production requires cell-cell contact with activated monocytes (223). Our study therefore reveals similarities and differences between mice and human regarding differentiation of Th17 cells.

3.2 Th17 cell effector mechanisms

Th17 cells seem to contribute to inflammation in several diseases through secretion of cytokines that induce pro-inflammatory mediators in cells of the affected tissues. In agreement with this, we found that *in vitro*-differentiated Th17 cells potently induce production of the pro-inflammatory cytokines IL-6 and IL-1β in primary bronchial epithelial cells. In addition, we observed up-regulation of G-CSF, GM-CSF and IL-8 production, consistent with their role in granulopoiesis and attraction of neutrophils (143, 218). To a certain extent, induction of these mediators was also observed in epithelial cells co-cultured with other T helper cell subsets, especially with Th1 cells. However, the effect was much less pronounced compared to Th17 cells. These results clearly show that *in vitro*-differentiated

Th17 cells differ from other T cell subsets in their potency to induce pro-inflammatory cytokines.

Th17 cells induced higher levels of IL-6 than equal amounts of recombinant IL-17A. Furthermore, the induction of IL-6 by Th17 cells was not fully abrogated by anti-IL-17A antibodies. Therefore, we speculate that other cytokines secreted by Th17 cells, such as IL-6, IL-22, IL-26, IL-17F or TNF-α (92, 96-99), synergize with IL-17A in the induction of pro-inflammatory cytokines in epithelial cells. Furthermore, the heterodimeric cytokine formed by IL-17A and IL-17F might be of relevance, as it has been shown to induce airway neutrophilia in mice (238).

IL-6 production by bronchial epithelial cells also substantially increased when the cells were stimulated with cell-free supernatants of Th17 cell cultures, suggesting that cell-cell contacts are not necessary for the induction of IL-6. Th17 cells produce more IL-17A when co-cultured with bronchial epithelial cells, indicating that Th17 cells promote the differentiation and expansion of their own population through induction of pro-inflammatory cytokines like IL-6 and IL-1β. Such a positive feedback mechanism is observed for other T helper cell subsets as well.

Elevated levels of both IL-6 and IL-17A have been found in plasma and airways of asthmatic individuals (213, 217). Our data showing that *in vitro*-differentiated Th17 cells potently induce IL-6 production by primary bronchial epithelial cells provide a further link between these cytokines and are a hint that Th17 cells contribute to airway inflammation in severe asthma. Through induction of neutrophil-attracting factors such as G-CSF, GM-CSF and IL-8, they might also play an important role in airway neutrophilia during allergic airway inflammation.

Taken together, our results show that human Th17 cells potently induce pro-inflammatory and chemoattracting mediators in primary bronchial epithelial cells and therefore indicate a role for Th17 cells in allergic asthma.

3.3 Expression of the transcription factor RORC2

The transcription factor RORC2 has been shown to play a key role in Th17 differentiation (162, 163), as RORC2-deficient mice show markedly reduced numbers of Th17 cells. Consistently, we found high levels of RORC2 in Th17 cells and substantially decreased IL-17 secretion after siRNA-mediated knockdown of RORC2. We also observed high levels of RORC2 in $CD8^+$ cells, which were reported to express IL-17A (220, 221). Two very recent reports confirm our finding of RORC2 expression in these cells (239, 240). Interestingly, we found high RORC2 levels also in NKT cells, consistent with a study reporting their IL-17 expression (222). In the course of this thesis, RORC2 has been described to play an important role in the development of an IL-17-producing subset of invariant NKT cells (241).

Surprisingly, we detected RORC2 to be expressed also in TGF-β-induced Treg cells, as well as in naturally occurring $CD25^+$ Treg cells, which did not produce IL-17A. This suggests that RORC2 expression does not necessarily imply IL-17A production. IL-17A expression in Treg cells might be suppressed by FOXP3, which we detected only in Treg, but not in Th17 cells. Such a repression has been suggested by two reports showing that in mice Foxp3 inhibits RORC2 function through direct interaction (14, 15).

Taken together, our findings indicate that RORC2, despite its crucial role in Th17 development, is not a selective marker for IL-17-producing T helper cells. Investigation of Th17 cells therefore requires analysis of other characteristic genes like for example their hallmark cytokines IL-17A and IL-17F.

3.4 Regulation of the *foxp3* gene by RORC2

During T cell differentiation, an individual T cell subset promotes not only its own development and expansion, it also inhibits differentiation of other subsets. Mutual inhibition of T cell subsets, which is an important mechanism of T cell polarization, is partially mediated by cytokines, but also by the lineages specific transcription factors. T-bet, the transcription factor of Th1 cells, has been shown to inhibit Th2 development (10), while

GATA-3 suppresses both Th1 and Treg cells differentiation (12, 13). FOXP3 inhibits transcriptional activity of RORC2, thereby limiting Th17 development (14, 15). In this thesis, we demonstrate that RORC2 is involved in T cell cross-regulation. Over-expression of RORC2 in naïve T cells led to a decreased expression of FOXP3 on both mRNA and protein level. Conversely, siRNA-mediated knockdown of RORC2 enhanced FOXP3 expression. The inverse correlation of the transcription factors suggests that the Th17 cell master regulator RORC2 negatively regulates expression of the Treg-specific transcription factor FOXP3. Physical interaction of RORC2 with the *foxp3* gene is necessary for RORC2-mediated repression, as overexpression of RORC2 lacking the DNA-binding domain did not inhibit FOXP3 expression. Moreover, no decrease in luciferase activity was observed upon co-transfection with the DBD-deficient mutant of RORC2. This is in agreement with the fact that DNA-binding dependent mechanisms of repression are commonly observed for ROR-family transcription factors.

These data reveal a novel function for RORC2 in T cell polarization. It remains to be determined whether RORC2 also influence the transcription factors of Th1 and Th2 cells and therefore represents a universal regulator of polarization.

3.5 RORC2 binding motifs in the FOXP3 promoter

Nuclear receptor family transcription factors mediate transcriptional activation or repression by binding to specific DNA motives in the regulatory region of their target genes. The consensus DNA binding elements for ROR-family members are ROREs. They consist of the DNA sequence GGTCA as a core motive, which is often preceded by an A/T-rich sequence (171, 172). In contrast to other nuclear receptors, members of the ROR-family transcription factors bind DNA as monomers. Therefore, their cognate DNA binding sites exist as single motives instead of palindromic sequences. We identified four putative binding sites for ROR family transcription factors in the FOXP3 promoter sequence from bp -1242 until bp +223, all consisting of single independent motives. Three of these ROREs are located in a poorly characterized part of the FOXP3 gene, slightly upstream of the core promoter and the region, which we previously identified as an inductive area for TCR-inducible transcription factors,

such as NFAT and AP-1. A fourth RORE is situated downstream the transcription start site. RORC2 was observed to bind to RORE-942 and to a lesser extend to RORE+115. Consistent with its importance in RORC2 binding, an A/T-rich sequence is found upstream of RORE-942. Also RORE-1096, to which we did not observe binding of RORC2, is adjacent to such a sequence. However, RORE-1096 is inverted, which might be a hint that it does not take part in RORC2-mediated inhibition and is therefore not interacting with RORC2. In contrast, no A/T-rich sequence is found upstream to RORE-847, consistent with our finding that it is not bound by RORC2. As gene regulation can take place in regions more distant from the transcription start site, it is likely that further ROREs exist, which were not analyzed in this study. Moreover, it remains to be determined, which of the ROREs that we found to interact with RORC2 are important for inhibition of the *foxp3* gene. Altogether, these experiments reveal that negative regulation of FOXP3 by RORC2 involves physical binding of RORC2 to two ROREs on its promoter, a mechanism commonly observed for ROR-family members.

3.6 Mechanisms of RORC2-mediated FOXP3 suppression

Transcription factors of the ROR-family exert their repressive function through interaction with co-repressors. However, very little is known about the mechanisms of RORC2-mediated gene repression. Likewise, the co-repressors with which RORC2 interacts to exert the negative regulation of the *foxp3* gene observed in this study still remain to be determined.

RORC2 has been shown to compete with NFAT for DNA binding, which leads to an inhibition of NFAT-induced gene transcription (229). As NFAT is important for TCR-induced activation of the *foxp3* gene (74), RORC2 might inhibit FOXP3 expression by competition with NFAT for its binding sites on the promoter, rather than through interaction with a co-repressor. However, in our experiments, we did not observe binding of RORC2 to NFAT binding sites in the FOXP3 promoter, which suggests that in the case of FOXP3, a different mechanism of inhibition takes place.

RORC2 has been reported to suppress IL-2 production (170). Consistent with this report, we observe an increased production of IL-2 in cells treated with siRNA against RORC2. IL-2

plays an important role in the development of Treg cells and in the induction of FOXP3 (76, 77). Therefore, RORC2 might inhibit FOXP3 expression by depriving the cells of IL-2.
Taken together, several mechanisms of RORC2-mediated FOXP3 suppression are possible and further experiments are needed to elucidate the molecular basis of the inhibition.

3.7 Mechanisms regulating the balance of Treg and Th17 cell differentiation

Treg and Th17 cells both depend on TGF-β for their development, while Th17 cells additionally require a pro-inflammatory environment (150-152, 162, 228). Based on these data and on our results, we like to suggest a model in which FOXP3-mediated inhibition of Th17 cells dominates under silent conditions, while FOXP3 expression is suppressed by RORC2 during inflammation, allowing the development of Th17 cells (Fig. 23). In this model, the differentiation of Treg versus Th17 cell is constantly balanced. The Th17 cell-inducing cytokines IL-6, IL-21 and IL-23 favor RORC2 over FOXP3 expression (90-92, 227, 234). The cytokine IL-27, in contrast, seems to favor the development Treg cells, as one of our previous studies showed that IL-27 enhances TGF-β-induced FOXP3 expression in a STAT1-dependent manner (82). Furthermore, IL-27 has been reported to inhibit development of Th17 cells (235-237). Retinoic acid is a key factor in regulating the balance of Treg versus Th17 cells (86-88, 231). It exerts its functions by binding to and thereby activating the retinoic acid receptor (RAR) α, its cognate receptor (89, 232). However, the mechanism of RA-mediated inhibition of Th17 cells might also involve RORC2, which is structurally closely related to RARα. Indeed, retinoic acid has been described to act as a ligand for RORC2, thereby inhibiting its transcriptional activity (178). Retinoic acid might therefore favor Treg cell development by binding and inhibiting RORC2, leading to an increased FOXP3 expression. However, in our study, addition of retinoic acid did not change extract-binding properties to the ROREs on the FOXP3 promoter, arguing for a different role for retinoic acid in the Treg-Th17 cell balance.

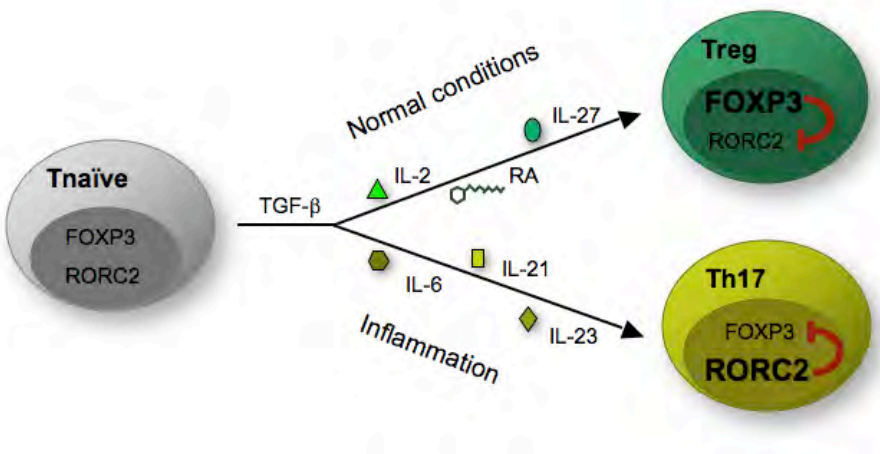

Fig 23. Factors regulating the Treg-Th17 cell balance. Under normal condition, IL-2, retinoic acid (RA) and IL-27 support FOXP3-mediated RORC2 inhibition, whereas inflammatory cytokines promote suppression of FOXP3 by RORC2 during inflammation. These mechanisms lead to the dominance of Treg cells and thereby to tolerance under silent condition, while ensuring predominance of Th17 cells during infection.

3.8 Phenotype shift upon RORC2 downregulation

Knockdown of RORC2 using siRNA increased FOXP3 level not only in a Treg-favoring environment, but induced comparably high levels of FOXP3 also in cells differentiated under Th17 conditions. These cells develop a phenotype characterized by high FOXP3 expression and low levels of pro-inflammatory cytokines. As previously reported (162, 163), IL-17A production was markedly reduced in Th17 cells that underwent siRNA-mediated RORC2 knockdown. Furthermore, we observed a reduction of other Th17 cell-related cytokines like IL-1β and IL-6. Interestingly, also the levels of IFN-γ and TNF-α, cytokines generally associated with Th1 cells, were lower in siRNA-treated cells, while the Th2 cytokines IL-5 and IL-13 remained unchanged. Knockdown of RORC2 therefore induces a downregulation of cytokines mediating inflammation.

Reduction of IL-17A expression is presumably a direct consequence of a lack of RORC2, as IL-17A expression is regulated by RORC2 on transcriptional level (167). The decrease in

IFN-γ after RORC2 knockdown, however, might be caused rather indirectly through increased FOXP3 expression, as FOXP3 has been shown to repress IFN-γ production (233). The fact that down-regulation of RORC2 shifts T cells from a pro-inflammatory towards a Treg like cell suggests RORC2 to be a target for therapies aiming to modify the inflammatory nature of an immune response and to induce tolerance.

3.9 Conclusion

This thesis investigated development and function of human Th17 cells and analyzed mutual cross-regulation of Th17 and Treg cells during T cell differentiation. We identified mediators inducing development of naïve human T cells into Th17 cells and showed that these cells significantly differ from other T helper cells in their potency to induce secretion of pro-inflammatory cytokines in primary bronchial epithelial cells. Furthermore, we revealed a role for RORC2 in T cell polarization through interaction with the FOXP3 promoter. T cells with down-regulated levels of RORC2 develop a Treg-like phenotype even under Th17-favoring conditions, suggesting that the role of RORC2 in Th17 cell development involves not only induction of Th17-characteristic genes, but also suppression of Treg-specific programs.

The current thesis reveals novel mechanisms of T cell polarization of subsets with opposed functions and thereby gives insight into the regulation of immunity and tolerance. These mechanisms are of considerable interest, as disruption of the balance between immunity and tolerance can lead to insufficient immune responses or inflammatory diseases. Our results therefore build a basis for the elaboration of therapies aiming to restore the balance between tolerance and immunity and suggest RORC2 to be a target for anti-inflammatory drugs.

4 CURRICULUM VITAE

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EDUCATION

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2001-2006:	**Master in Biochemistry and Molecular Biology ETH Zurich, Zurich, Switzerland** Subjects: Immunology, Biochemistry, Molecular Biology, Gene and Enzyme Technology, Bioorganic Chemistry
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5 PUBLICATIONS

Burgler S, Mantel P-Y, Bassin C, Ouaked N, Akdis CA, and Schmidt-Weber CB "RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter" *Submitted.*

Meyer N, Zimmermann M, **Burgler S**, Bassin C, Woehrl S, Moritz K, Rhyner C Indermitte P, Basinski T, Schmidt-Grendelmeier P, Akdis M, Menz G, Akdis CA "IL-32 controls apoptosis of keratinocytes and eczema formation in atopic dermatitis" *In revision.*

Burgler S, Ouaked N, Bassin C, Basinski T, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB. "Differentiation and functional analysis of human T helper 17 cells" *J Allergy Clin Immunol.* 2009, 123: 588-95.

Siegmund K, Ruckert B, Ouaked N, **Burgler S**, Akdis CA and Schmidt-Weber CB. "Unique Phenotype of Human Tonsillar and In Vitro-Induced $FOXP3^{+}CD8^{+}$ T cells" *J. Immunol*. 2009, 182: 2124-30.

Ouaked N, Mantel P-Y, Bassin C, **Burgler S**, Siegmund K, Akdis CA, and Schmidt-Weber CB. "Regulation of the *foxp3* Gene by the Th1 Cytokines: The Role of IL-27-Induced STAT1" *J. Immunol.* 2009, 182: 1041-9.

Hasan S, Guttinger S, Muhlhausser P, Anderegg F, **Burgler S**, Kutay U. "Nuclear envelope localization of human UNC84A does not require nuclear lamins." *FEBS Lett.* 2006; 580(5): 1263-8

6 PRESENTATIONS

6.1 Presentations at Congresses

6.1.1 Oral Presentations

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Differentiation and functional analysis of human Th17 cells". 7th EAACI-GA^{2}LEN Immunology Winter School, Davos, Switzerland, 7rd February 2009

Burgler S, Ouaked N, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Th17 Zellen in Krankheit". Klinische Konferenz, Davos, Switzerland, 2nd December 2008

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Development and function of human Th17 cells". Young scientists in contest, Davos, Switzerland, 28th August 2008

Awarded with the "Best Presentation Award" sponsored by UBS

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Development and functional analysis of human Th17 cells". Medical Research Council-Asthma UK Centre Retreat, Wye, UK, 7th September 2007

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Human Th17 cells express the TGF-beta-dependent transcription factor RORC2". 5th EAACI-GA^{2}LEN-Davos meeting, Davos, Switzerland, 3rd February 2007

6.1.2 Poster Presentations

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Differentiation and functional analysis of human Th17 cells". World Immune Regulation Meeting III, Davos, Switzerland, 22th March 2009

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Development and function of human Th17 cells". World Immune Regulation Meeting II, Davos, Switzerland, 19th March 2008

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Meyer N, Akdis CA, and Schmidt-Weber CB "Development and function of human Th17 cells". 6th EAACI-GA^{2}LEN-Davos meeting, Pichl, Austria, 2nd February 2008

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Akdis CA, and Schmidt-Weber CB "Human Th17 cells express the TGF-β-dependent transcription factor RORC2". World Immune Regulation Meeting I, Davos, Switzerland, 13th April 2007

Burgler S, Ouaked N, Bassin C, Mantel P-Y, Siegmund K, Akdis CA, and Schmidt-Weber CB "Human Th17 cells express the TGF-beta-dependent transcription factor RORC2". XIX. Meeting of the Swiss Immunology Ph.D. students, Schloss Wolfsberg, Ermatingen Switzerland, 26th March 2007

6.2 Promotion Committee Meetings

Burgler S "Development and function of human T helper 17 cells" Promotion Committee Meeting, Zurich, Switzerland, 8th October 2008

Burgler S "Development, phenotype and function of human Th17 cells" Promotion Committee Meeting, Zurich, Switzerland, 6th August 2007

6.3 Progress Reports

Burgler S "Differentiation and function of human Th17 cells and the role of their master regulator RORC2 in T cell polarization" Progress Report at Swiss Institute of Allergy and Asthma Research, 13th October 2009

Burgler S "Regulation of FOXP3 by the transcription factor RORγt/RORC2" Progress Report at Swiss Institute of Allergy and Asthma Research, 26th Mai 2009

Burgler S "Regulation of FOXP3 by the transcription factor RORγt/RORC2" Progress Report at Imperial College London, 14th Mai 2009

Burgler S "Development and function of human Th17 cells" *Science Day* at Swiss Institute of Allergy and Asthma Research, 16th December 2008

Awarded with the "Best Presentation Award"

Burgler S "Development and function of human T helper 17 cells" Progress Report at Swiss Institute of Allergy and Asthma Research, 28th October 2008

Burgler S "TGF-β contributes to the differentiation of human Th17 cells by inducing their transcription factor RORC2" Progress Report at Imperial College London 19th May 2008

Burgler S "TGF-β contributes to the differentiation of human Th17 cells by inducing their transcription factor RORC2" Progress Report at Swiss Institute of Allergy and Asthma Research, 14th April 2008

Burgler S "Development and function of human Th17 cells" *Science Day* at Swiss Institute of Allergy and Asthma Research, 13th November 2007

Burgler S "Human Th17: role of RORC2 in their development and influence of Th17 on the epithelium" Progress Report at Swiss Institute of Allergy and Asthma Research, 25th September 2007

Burgler S "Human Th17 cells express the TGF-β-dependent transcription factor RORC2" Progress Report at Swiss Institute of Allergy and Asthma Research, 6th March 2007

Burgler S "Differentiation of naïve T-cells to Th17 cells " Progress Report at Swiss Institute of Allergy and Asthma Research, 26th September 2006

6.4 Journal Clubs

Burgler S "Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay" Journal Club at Swiss Institute of Allergy and Asthma Research, 28th April 2009

Burgler S "Tyrosine-phosphorylation-dependent translocation of the SLAT protein to the immunological synapse is required for NFAT transription factor activation" Journal Club at Swiss Institute of Allergy and Asthma Research, 2th December 2008

Burgler S "TGF-β-induced Foxp3 inhibits Th17 cell differentiation by antagonizing RORγt function" Journal Club at Swiss Institute of Allergy and Asthma Research, 29th May 2008

Burgler S "Deacetylase inhibition promotes the generation and function of regulatory T cells" Journal Club at Swiss Institute of Allergy and Asthma Research, 27th November 2007

Burgler S "Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation" Journal Club at Swiss Institute of Allergy and Asthma Research, 16th May 2007

Burgler S "Spatiotemporal regulation of c-Fos by ERK5 and the E3 ubiquitin ligase UR1 and ist biological role." Journal Club at Swiss Institute of Allergy and Asthma Research, 28th November 2006

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